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*Stability of a translocated chromosome in
Chronological Ageing Yeast*

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A Thesis Submitted in Fulfillment of the Requirements
of the Open University, (UK) for the Degree of Master of
Philosophy

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Abstract

Chromosome translocation is a disastrous event for a cell, affecting almost every aspect ranging from metabolism, organelle maintenance and homeostasis to gene maintenance and expression. By using the Bridge Induced Translocation (BIT) system I wanted to define the effects of an induced translocation on the Chronological Life Span (CLS) of yeast with particular interest to the maintenance of the translocated chromosome through-out the life span. The results demonstrate that every translocant strain has a different CLS with a high increase in Reactive Oxygen Species (ROS) levels at the end of the life span but the loss of the translocated chromosome occurs at the end of the life span with different rates between the translocants. Furthermore, the *RDH54* gene may play a role in the correct segregation of the translocant chromosome since in its absence there is an exponential increase in loss of that chromosome.

1. Introduction

1.1. Cell cycle and chromosome dynamics

There are many mechanisms that regulate mitosis and meiosis in yeast and in the past decade there have been significant advances in this field. Molecular motors that move chromosomes have been identified, the microtubule assembly/disassembly and the movement of the chromosomes has been more clearly defined. But it has been much more difficult to determine the mechanism of chromosome condensation and sister chromatid cohesion, meaning how they remain together after DNA replication until they separate at anaphase.[5]

The duplicated chromosomes (the sister chromatids) to be segregated with fidelity must have a mechanism to ensure that each partner in pair 'knows' where the other is when the decision to segregate is made.

A brief description of the eukaryotic cell cycle must be made in order to comprehend the dynamics of the chromosomes. The eukaryotic cell cycle consists of two separable phases, interphase and mitosis. In interphase three sections are distinguishable (G1-, S-, and G2-phase) whereas mitosis comprises four sections: Prophase (chromosome condensation); metaphase (chromosome alignment); anaphase (chromosome separation); and telophase (chromosome decondensation). For the cell to operate correctly there are three checkpoints that it must pass. The first is localized at the end of the G1 phase, the second at the late G2 phase and the third and last before anaphase. Before starting, cells can choose between entering the mitotic cycle, providing they have all the necessary nutrients and they have

reached their critical size, or initiating the sexual reproduction proving if they are in a starvation condition. During G2-phase, cells have to be sure that DNA replication has been achieved without causing any damage to the DNA. The entrance to anaphase depends on the correct chromosome alignment and proper spindle formation. If these checkpoints fail or if the order of the events is incorrect, cell division will lead to genetically aberrant progeny. [6]

1.1.1. Segregation

Chromosome separation is a highly delicate and irreversible process and must be therefore regulated. Unfortunately once the sister chromatids are aligned they cannot be repaired, in mitosis but not in meiosis, by recombination and furthermore aberrant chromosomes cannot be dis-attached once they are aligned on the metaphase plate.

It is important to note that sister chromatids are attached one to another by a multisubunit complex called cohesin that binds to chromosomes at multiple sites from telophase to the onset of the anaphase in the next cell cycle. Cohesins are formed by 4 subunits named Smc1p, Smc3p, Scc1p and Scc3p. Smc1p and Smc3p form the SMC complex where at one end there is a globular hinge domain whereas at the other end there is an ABC-like ATPase head domain. Scc1p and Scc3p, instead, heterodimerize via their hinge domain; the kleisin subunit Scc1p connects their ATPase heads to the Scc3p.

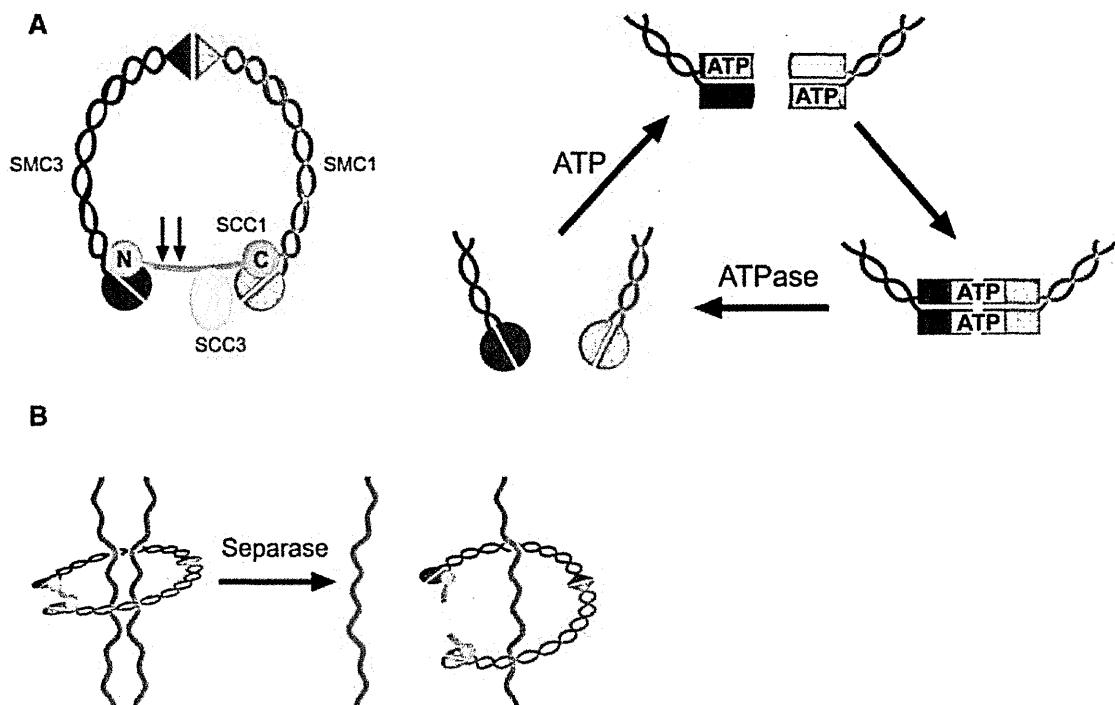


Figure 1: A, schematic structure of the SMC3 (blue) and SMC1 (red) that form a coiled coil by uniting at their ATP ends (triangles). The SCC1 (green) COOH end binds to the SMC1 ATPase head whereas the NH₂ terminus binds to the SMC3 head. The SCC3 binds to the SCC1 COOH-terminal half and does not make contact with the NH₂ part. The arrows mark the region in which the separase acts. It is assumed that by ATP binding or hydrolysis there is the opening or closing of the cohesin ring. B, Cohesin keep the two sister chromatids together by trapping them inside the ring. Picture readapted from reference [1]

This suggests that cohesins form a ring in which the DNA strands are trapped, thereby it was concluded that the connection between the sister chromatids must be a topological rather than a chemical one.[1]

On the start of the anaphase the cleavage of the Scc1p is performed by a cysteine protease known as separase and it acts by disrupting the interaction between the SMC heads in cohesion enabling the ring to be opened. Furthermore it has recently been discovered that the Scc1p and the Scc3p not only connect the Smc3p and the Smc1p but also regulate their ATPase activity.[7-9]

There is a second chromatid stabilizing complex called condensin. It consists of a 13S complex with two SMC proteins: Smc2p and Smc4p, contains three other essential subunits, one of which is homologous to Scc1p. Condensin has the capacity to associate with chromatin independently of ATP but its hydrolysis is needed for a binding reaction. A feature worth of note is that chromatin wraps around it generating torsion in the DNA.[10] Therefore condensin contributes to chromosome compaction and it also participates in DNA repair. After the removal of the cohesin rings by the separase reaction, condensin replaces cohesin, both in mitosis and in meiosis.[11, 12]

The segregation and movement of the chromatids to the opposite poles of the cell is triggered by the fluctuation of the mitotic cyclins during the cell cycle. At the late G2-phase when the anaphase commences the CDK activity is destroyed by the degradation of the cyclins, but it is not required for the separation of the sister chromatids. The apparatus responsible for this degradation is a highly conserved multisubunit complex that possesses ubiquitin ligase activity and it is named APC

(anaphase promoting complex). In addition, it mediates also the degradation of other protein components, other than cyclins, necessary for the separation of the sister chromatids [13-18].

To ensure a correct nuclear division between the mother and the daughter cells and to have a correct sister chromatid separation, the mitotic spindle must be oriented along the cell polarity axis defined by the site where the bud emerges.

The spindle pole is essential for cell cortex regulation of the cytoplasmic microtubules attachments during spindle morphogenesis. Among the cortical components implicated in spindle orientation are Bni1p that is target of the polarizing machinery essential for bud site selection and spindle orientation, and the actin interactor Aip3p/Bud6p localized in the bud tip [19-21]. The spindle morphogenesis in yeast is initiated at the G1-S transition of the cell-cycle. Progression through the START triggers bud emergence, DNA replication, and the duplication of the microtubule-organizing center (MTOC)-the spindle pole body (SPB). [22] The polymerization and depolymerization of tubulin and the microtubules' motility are assisted by mechanochemical enzymes or motor proteins that are necessary in spindle morphogenesis: the cytoplasmic dynein and the kinesin-like proteins Kip2p and Kip3p, as well as Kar3p, are involved in regulating microtubule dynamics and mediating nuclear migration to the bud neck and facilitating spindle translocation.[23] The microtubules emerge from the SPBs toward the new bud and orientate the nucleus and intranuclear spindle mitosis. The nuclear membrane in yeast remains intact (when in mammals it dismantles) throughout mitosis with the mitotic spindle forming intranuclearly between the two

SPBs embedded in the nuclear envelope (NE). Once the genome duplicates, the spindle aligns parallel to the mother bud axis and finally elongates to supply one nucleus to both mother and daughter.

Bim1p can directly bind to microtubules and is required for the highly dynamic instability of microtubules that is characteristic of cells before spindle assembly. Kar9p has been implicated in the orientation of functional microtubule attachments into the bud during vegetative growth. It is delivered to the bud by a Myo2-dependent mechanism. The interaction between Bim1p and Kar9p appears to provide a linkage between the actin and the microtubule cytoskeleton. Bud3p, a protein for axial budding of haploid cells, accumulates at the bud neck and is required for the efficient association of Bud6p with the neck region.

1.1.2. Spindle Checkpoint

The spindle checkpoint, that controls the activity of the Anaphase Promoting Complex (APC), is a surveillance mechanism shared by most eukaryotic cells, which prevents the separation of the sister chromatids when the spindles are damaged or the chromosomes fail to attach. The kinetochore of a single lagging chromosome emits a signal strong enough to stop the separation of all sister pairs. [24-26] The kinetochore itself is composed by three protein assemblies that can be divided in inner, central and outer. The outer complex DAM1, composed by Duo1p and Msp1p interacting complex, has been discovered to play a crucial role in mediating the kinetochore-microtubular connection and is regulated through phosphorylation by the Ipl1p/Aurora B kinase. The central complex instead is composed by *IPL1*, *CFT19*

NDC80 and *MWT1* that are associated to the microtubules via *DAM1* and to the kinetochores via the kinetochore inner complex.

The CBF3 (centromere binding factor 3) is formed by Ndc10p, Cep3p, Cft13p, and Skp1p, as well as a number of chromatin-specific proteins, which are required to build up a kinetochore at each centromere. It is the most critical complex since it recruits the rest of all kinetochore proteins.

The IPL1 complex responds to the lack of tension and acts to resolve the possible inappropriate attachments. In the absence of tension, Ipl1p causes an increased turnover of kinetochore-microtubule connections. Experiments have demonstrated that the tension resulting from the physical connection between bioriented kinetochores and the activity of Ipl1p is sufficient for the proper alignment of sister chromatids.[27] Once the chromosomes are successfully oriented there is a rapid disjunction of the sister chromatids at anaphase which requires the cleavage of cohesin subunit Scc1p by a cysteine protease called separase, that is inactivated throughout most of the cell cycle by its association with the inhibitor securin.[28-30]

The transition from metaphase to anaphase is initiated when securin is degraded by the proteasome as a consequence of ubiquitination by the multicomponent E3 ubiquitin ligase known as the APC/C. The function of the APC/C is regulated by phosphorylation and association of activator proteins such as Cdc20p and its homolog Hct1p, which modulates the affinity of the APC/C to different substrates.[31-33]

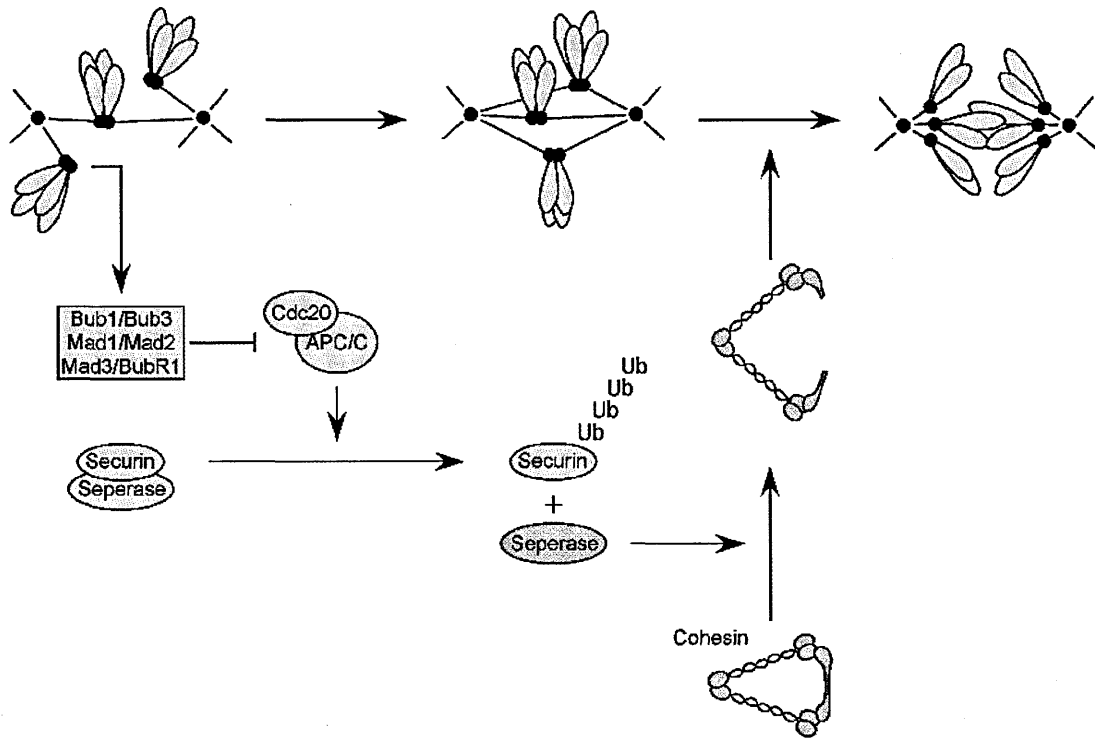


Figure 2: The APC/C is the downstream target of the spindle checkpoint. Unattached kinetochores activate the Mad/Bub-dependent checkpoint pathway which inhibits Cdc20 to activate the APC/C. When all the chromosomes align, the checkpoint signal is extinguished thus allowing Cdc20 to activate the APC/C. Securin is then polyubiquitinated, targeting it for proteolysis and thus releasing the Separase. Separase then cleaves the Scc1 subunit of Cohesin, opening the ring structure and thus allowing sister chromatids to separate. This cartoon was taken from reference [34].

The activation of the spindle checkpoint inhibits the ubiquitin-dependent proteolysis of securin by APC/C and it also blocks any further cell cycle progression. In yeast this progression is triggered by recruitment of a complex to the Spindle Complex, which contains the proteins Mad1p, Mad2p, Mad3p and the proteins kinases Bub1p and Bub3p, of which Bub1p is activated through Cdc2p. Bub3p binds to the activator Cdc20p of the APC/C complex and therefore block the ubiquitination of both securin and cyclin B. In addition, protein kinase Mps1p is also required for the spindle pole duplication as well as the subunit Ndc10p of the centromere binding complex CBF3. The Mad complex has been shown to be highly conserved among other eukaryotes. [35-38]

1.2. Homologous Recombination

Homologous recombination has been known to be the crucial mechanism for the correct repair of double strand breaks (DSB) and other DNA damages and it occurs between two DNA strands in G2. DSBs are formed in different ways such as: by radiation, chemical compounds and by blocked or broken replication forks. The basics of this mechanism are that once there is a DSB, a strand invasion of the broken chromosomes into the intact chromosome commences using it as a template for initiating the DNA repair. Once it has been repaired, DNA replication can continue. Furthermore the homologous recombination is used by the cell to exchange genetic information between different chromosomes in order to enhance the genetic variability in the chromosomes.[39] Homologous recombination has also a mechanical role in the segregation of the chromosomes during meiosis I.

Different mechanisms that have the same initial steps can be grouped under Homologous Recombination, of these Double Strand Break Repair (DSBR) and Synthesis-Dependent Strand Annealing (SDSA) will be dealt with in this thesis. The DSBR mechanism shares the first part of the processing of the repair with the SDSA. After the initial DSB there is a resection of one strand in order to produce an overhanging 3' strand that will invade the D-loop formed by the homologous dsDNA generating a first Holliday Junction (HJ) [40]. After this initial part the two mechanisms follow a different method: the DSBR forms a second Holliday Junction at the other end of the D-loop and repairs the break by DNA-synthesis and ligation. The double Holliday Junction can be resolved in two ways in a non-crossover or crossover manner depending on where the HJ is cut. On the other hand the SDSA proceeds by strand displacement and annealing followed by gap-filling DNA-synthesis and finally ligation. This method will always resolve in a non-crossover. The HR machinery is formed by the RAD52 epistasis group which is a class of recombinases. These proteins were initially defined in the budding yeast and are hypersensitive to any DNA-damaging agents such as ionizing radiation and DNA-crosslinking chemicals that eventually lead to DSBs formation. Mutation of any of these genes leads to DNA-damage sensitivity and defective HR. Of the RAD52 epistasis group Rad51p is the most closely related to the E. coli RecA and possesses a recombinase activity that links recombining chromosomes through DNA joint formation.[41, 42]

A. The double-strand-break repair model

B. Synthesis-dependent strand-annealing

C. Break-induced replication

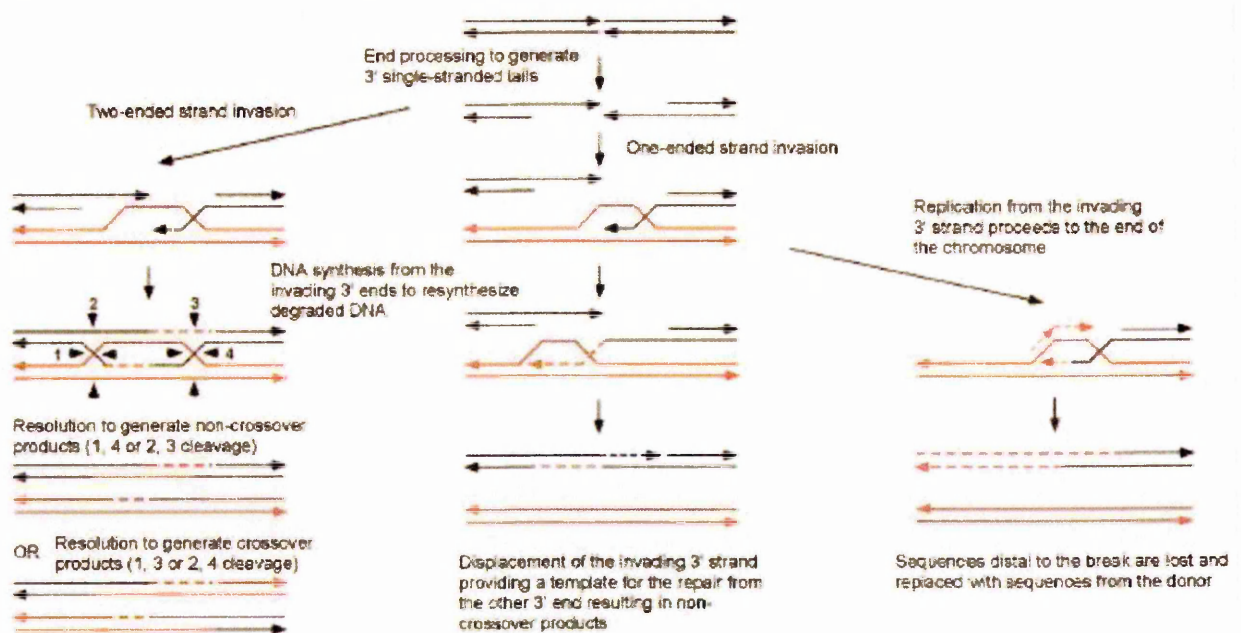


Figure 3: Schematic representation of the different homologous recombination systems, including the Break Induced Replication. Figure re-adapted from reference [4]

The substrate of the HR machinery is the ssDNA that comes from the processing of the DSB or another lesion. Its function is to recruit Rad51 and its associated factors. *RAD52* forms a highly regulated and ordered right handed helical structure that wraps around the ssDNA and interacts physically with Rad51p by nucleating Rad51p onto a RPA-coated ssDNA template to facilitate the pre-synaptic filament assembly. Once Rad51p and Rad52p are on the pre-synaptic filament, the complex RAD55-RAD57 will also interact with the DNA assembly in order to further stabilize the complex and to block the inhibitory effect of the pre-synaptic assembly given by RPA. RAD55-RAD57 is a heterodimer that physically interacts with ssDNA coated with Rad51p.

When the pre-synaptic filament is assembled Rad54p will bind to it and mediate the search of the homologous sequence just to, afterwards, provide the strand invasion hence forming the D-loop. Rad54p is the critical factor in the strand invasion since it will remodel the chromatin in order to facilitate the DNA joint formation.[4] The resolution of the HJ, in the end, is mediated by a topoisomerase I, that depending on where it cuts it can resolve the HJ in a non-crossover or crossover matter.

1.2.1. Break Induced Replication

The Break Induced Replication (BIR) follows a different path to repair the broken chromosome. It initiates in the same way with the resection of the lagging strand in order for it to be coated by Rad51p, after which it mediates strand invasion with the establishment of a processive replication fork with the synthesis of the DNA that proceeds to the end of the chromosome. The strand invasion step is the same as

the DSB mechanism involving the Rad51p, Rad52p, Rad54p and RAD55-RAD57 complex but the result and timing is different due to the fact that BIR requires a leading and lagging strand that will result in a LOH (loss of heterozygosity).

In addition the main difference is that the process initiates only when one end of the DSB of a chromosome shares homology with a template and the lagging strand recruits Pol α -primase complex and Pol δ , with the presence of the subunit Pol32p, to initiate DNA synthesis. In fact it is believed that it cuts at the eroding telomere ends to reestablish their length and to heal collapsed or stalled replication forks.[43, 44]

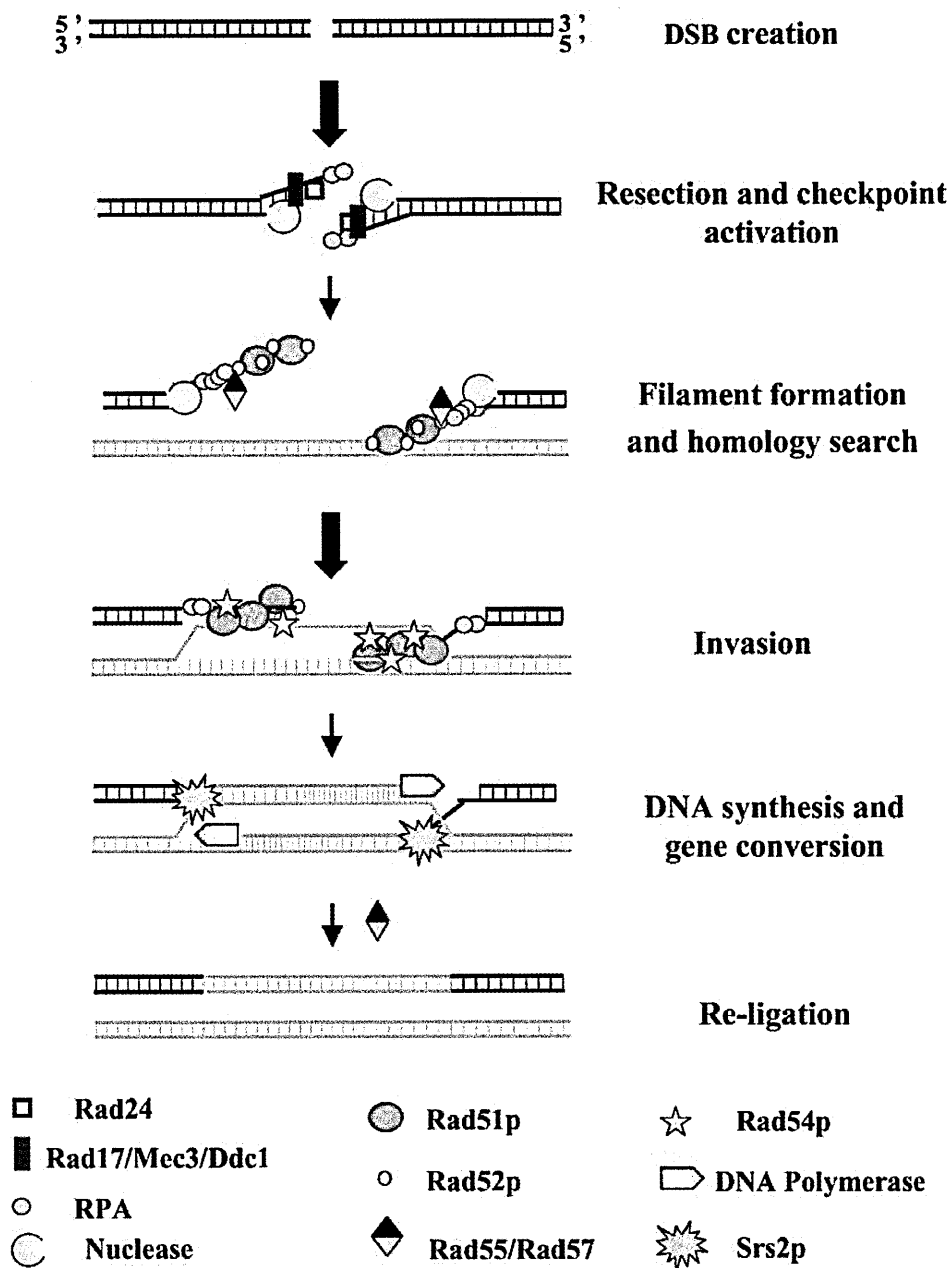


Figure 4: Cartoon representing the homologous recombination system with the proteins involved at the different steps. Figure re-adapted from reference [2]

1.3. Chromosomal Translocations

Mitotic DSBs double strand breaks that form due a variety of events ranging from replication fork stalling to ionizing radiation, can lead to unbalanced reciprocal or non-reciprocal chromosomal translocations causing loss of heterozygosity (LOH) for tumor suppressor genes [45, 46], or to reciprocal translocations resulting in gene fusions. In some lymphomas and leukemias, chromosome translocations lead to the juxtaposition of promoter/enhancer regions of one gene with the intact coding region of another gene. In contrast, translocations in the chronic myeloid leukemia and in other chronic acute leukemias, the translocations results in the recombination of coding regions of two different genes. This results in a fusion protein that might have a new function. This is the case of the BCR-ABL fusion protein that is encoded by the Philadelphia chromosome. [47]

The molecular pathway leading from the DSBs to the formation of the chromosomal translocation is still unclear, but the extensive study done on the yeast *Saccharomyces cerevisiae* is leading us closer in understanding the molecular pathways of the DSBs repair and suppression of the genomic instability. [48, 49]

In yeast it has been demonstrated that a chromosomal DSB produced by a HO endonuclease could be repaired by the BIR system resulting in a chromosomal translocation [50]. Additionally a recent *cre*-specific recombination site system producing reciprocal translocations at pre-engineered *loxP* sites has been developed. [51]

1.4. Bridge Induced Translocation

The Bridge induced translocation system has been developed in the Bruschi laboratory at the ICGEB Trieste and it is a novel system to induce a chromosome translocation using homologous sequences and it can be done between heterologous or homologous chromosomes.

The idea is to introduce inside the cell a bridge harboring on both ends the sequence of integration for a specific locus and in the center a resistance marker to select the cells that are positively transformed. [52]

The integration of the cassette can occur in two different ways depending on the position of the locus targeted, as a matter of fact if the targeted locus is in the sub-telomeric region the integration of the bridge will be via the BIR (break induced replication) mechanism whereas if it happens in non-sub-telomeric regions the integration will be initiated by the DSBR mechanism. It has been shown that *RAD54* plays a crucial role in the BIT integration and that *RDH54* plays an important role not only in the stability of the translocated chromosome in mitosis (translocation between heterologous chromosomes) but also in the translocation between homologs where it seems to be involved in the Holliday junction resolution.

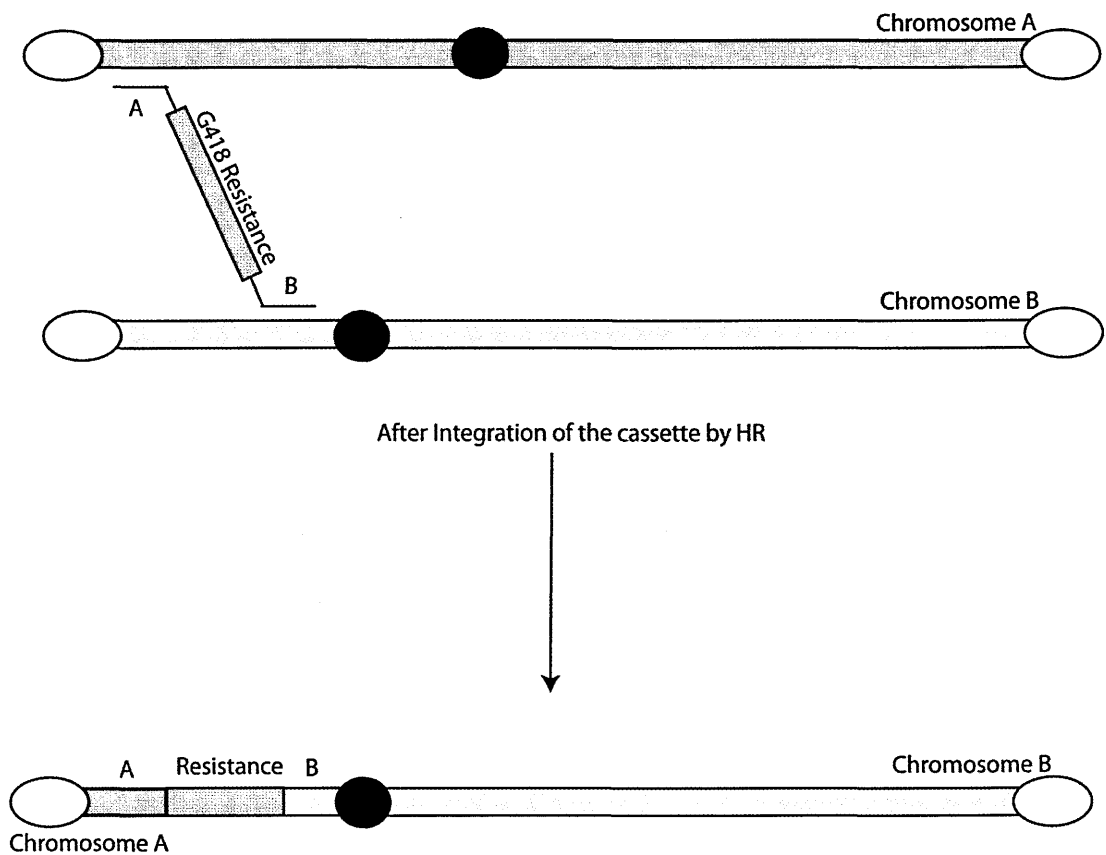


Figure 5: The schematic representation shows that after inserting a cassette harbouring a homologous sequence for chromosome A and a homologous sequence for chromosome B with in the middle a selective marker, in this case the resistance to G418., the cell by integrating the cassette on both ends induces the production of a translocated chromosome.

BIT is still commencing in the *pol32Δ/pol32Δ* strains indicating that the event to complete the replication is *POL32* independent [53].

There is much more to the BIT event than a simple translocation and the mechanism behind it, in fact it has been demonstrated that many rearrangements arise in the cell, such as multiple aneuploidies, after the same bridge induced translocation [54]. Furthermore the expression of the genes harboring near the breaking point, the actin network regulation and the ROS levels are modified, in addition it has been confirmed that chromosome translocation may also lead to an increased drug resistance [55] (Nikitin D. 2012 in press)

The BIT system can also be implied for a translocation between homologous chromosomes inducing a LOH of approximately 40kb [56], this shows the multiple features of this simple but at the same time complicated system.

Thanks to the BIT system it is possible to show that the effect of a translocation is not only localized to the loci involved but it also affects the cell on the whole. In fact it has been shown that translocation has a double effect on the chronological life span of the cell either increasing or decreasing it depending on the chromosomes involved and on the locus targeted [57].

If we look deeper in the mechanism of BIT and how the integration of the cassette occurs it is possible to identify that not only the HR mechanism influence on the efficiency of the BIT but also the length of the cassette and the loci targeted, in addition it has been proved that loci that have the same distance between them, three dimensionally speaking, have a better efficiency of translocation if the length of the bridge chosen is the exact distance between the two loci[58].

1.5. Ageing

Ageing and its processes has always been one of the mysteries of science and scientist have always tried to unravel its mechanism. Yeast has been the most important model for the study of ageing since it is very easy to manipulate and keep in culture, but most importantly it is possible to actually count and separate the mother cell from the daughter cell and analyze them separately, furthermore it has been show that a mother cell can divide a certain number of times before it enters senescence. These important features have made yeast the main model for the study of ageing and it has given scientists the possibility to better understand the mechanism behind it.[59] Yeast can provide us with two independent ageing models that are similar to the ageing processes in mammals but do not share between them the same genes and pathways.[60]

These two methods are known as: Replicative Life Span that measures how many times a mother cell can produce a daughter cell before entering senescence and Chronological Life Span that measures the mean and maximum survival of non-dividing yeast after reaching stationary phase in synthetic complete media.

1.5.1. Chronological Ageing

The chronological life span method has been developed by Valter Longo of the University of Los Angeles and has been since then widely used as a model to study how cells that don't divide, age. It has been shown, as previously cited, that Chronological Life Span doesn't share completely the same pathway as the

Replicative Life Span but some of its players have a similar and sometimes opposite role.

The CLS seems to be particularly well suited to examine and identify conserved life span mediators because the measurements are performed under the conditions that may have been present when the longevity pathways have evolved. [61]

From a technical point of view the CLS assay is very simple to perform and doesn't require any micromanipulation, in fact the cells are left to grow in a media and then after a certain amount of days, a sample is picked and plated on rich media to count the Colony Forming Units (CFU). With the increasing of the days there is a decrease in the CFU and therefore it is possible to draw a life span chart.

The pathways that control CLS have yet to be entirely discovered but its main players are known, in fact Sch9p, Tor1p and Ras2p are at the top of these pathways. Sch9p most importantly has been shown to dramatically enhance the life span if deleted in yeast cells. [62]

There are two major pathways that control CLS and RLS, the TOR/Sch9 and the Ras/adenylate cyclase/PKA pathway. To enhance the CLS the expression of the two pathways must be lowered. Interestingly the mitochondrial superoxide dismutase activity (SOD2) if overexpressed it enhances the CLS but shortens RLS.[63] Vice versa while the additional copy of *SIR2* is able to elongate RLS it shortens CLS in cells lacking the *SCH9* that live 3 fold longer in normal context but 5 fold longer in cells that are $\Delta sir2$. [64]

It is possible, then, that the pathways and their downstream transcription factors affect ageing by similar mechanism on both RLS and CLS, but since RLS is based on

cell division and since protective enzymes and stress-resistance can negatively affect cell division, the cells would stop dividing in order to save themselves from severe damage or death.[65]

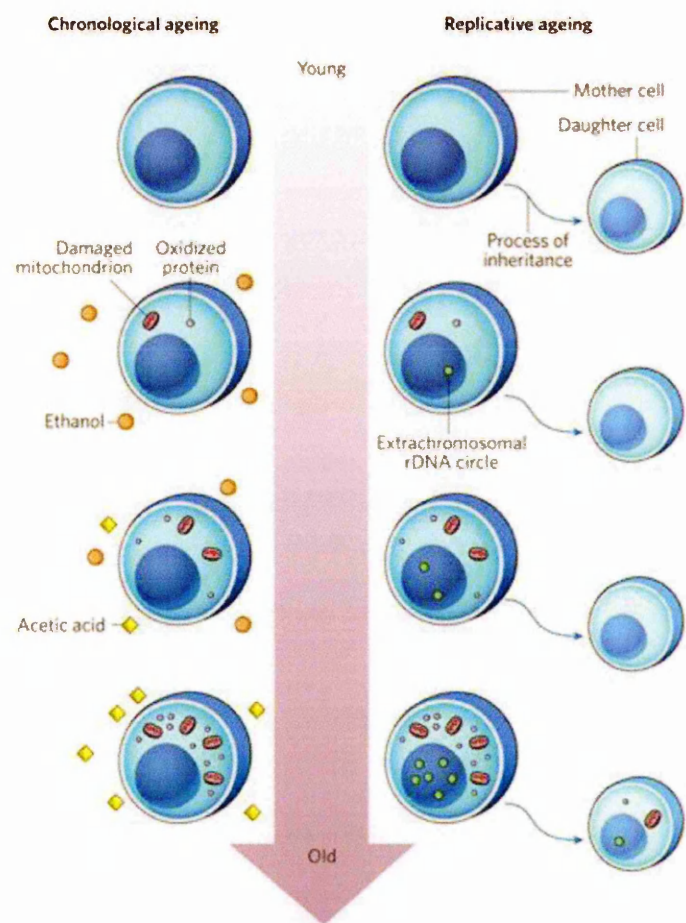


Figure 6: Cartoon representing Chronological vs. Replicative life span and their related DNA damage. Image adapted from [3]

1.5.2. Oxidative Stress in Ageing cells

In some cases life span extension has been associated with lower levels of Reactive Oxygen Species therefore with an increased ability to withstand stress. It is believed that ageing is caused by high levels of cellular damage caused by ROS that are mostly generated in the mitochondrial complexes I and III of the Electron transport chain. [66]

Experiments on the mitochondrial superoxide dismutase (SOD2) or on the combination of both SODs them have shown to increase up to 30% the CLS which is lower if compared to the 3-fold of *Δsch9* but still considerably high. Similar results have shown also in flies and mice suggesting that MnSOD provide a fundamental anti-oxidant defense but a rather limited role in the extension of the CLS. Nevertheless while ROS activity has been shown to have lower effect on the CLS itself, it has a major implication in the age-dependent formation of mutations that spread from the simple point mutation to the Gross Chromosomal Rearrangement. [67]

To what extent Genomic Instability contributes to ageing is still uncertain, but its role in tumorigenesis is well known and major evidence shows the intertwined relationship between oxidative DNA damage, mutations and cancer. In fact a high number of mutations shown in the oncosuppressor p53 that is mutated in approximately 50% of human cancers, are generated in the attempt to repair the damage caused by the reactive oxygen species.[68] In addition high levels of DNA damage/mutations and cancer have been found in mice lacking the cytosolic or

mitochondrial superoxide dismutase (SOD). [69] In concordance to these findings, also in yeast the lacking of the cytosolic Sod causes an increase in mutations and a high frequency of “adaptive regrowth”, a phenotype described as cancer-like since it is the ability of ageing cells to resume cell division under conditions that normally do not promote growth. [70, 71]

1.5.3. Genomic Instability in Chronological Ageing yeast

Before going into detail defining the different DNA damages that occur in chronologically ageing yeast, it is important to clarify any misunderstanding about the population that actually endures these mutations.

As described before, chronological ageing examines the survival or reproductive capacity of cells in stationary phase and it is often used as a model to study those cells that in a multicellular organism are non-dividing or postmitotic. Neither senescent nor quiescent cells divide but, while the quiescent cells are able to retain the ability to answer to growth signals, if the conditions are appropriate and thereby initiate mitogenesis, senescent cells cannot respond to these signals and therefore lose their ability to divide. It was recently discovered that loss of the reproductive capacity in stationary phase yeast cells precedes the loss of viability and therefore reflects the model of senescent cells. [72]

Another point of confusion is that it was assumed that stationary yeast cells reside in a uniform quiescent non-dividing state until they eventually die. But in reality a large fraction of cells in stationary phase cultures are not quiescent. [73]

This fraction of non-quiescent cells is even larger if the media used for the CLS experiment is defined medium, rather than rich medium. The explanation is the accumulation of acetic acid that occurs in the defined medium while cells enter stationary phase. [71, 74] This acetic acid accumulation lowers the pH in the defined medium and this lower pH activates the same growth signaling pathways triggered by glucose in yeast or glucose and mitogens in mammals. Thereby this particular situation promotes senescence and inhibits quiescence. [75]

An additional misunderstanding is that it is believed that when cells enter stationary phase they cease to divide, in reality, in defined medium, the fraction of cells in S phase first declines to 0% at day one of the CLS and then steadily rise with time in stationary phase [71]. Thus some cells in the defined medium appear to be re-entering the cells cycle probably due to the low pH provided by the acetic acid that increases the growth signaling, this re-entering the cells cycle is followed by a parallel increase in the non-quiescent fraction of stationary phase cells. This steady state turn-over of cells that during longer periods of time is represented by a larger fraction of cells that continue to divide and thereby promote the adaptive regrowth phenotype [71].

Now that most doubts have been cleared it is easy to explain that by performing simple mutation assays on chronological ageing yeast, it is possible to monitor age-dependent accumulation of different types of DNA mutations that spread from small DNA insertion/substitutions to GCRs. [76]

All these mutations increase in an age-dependent matter but a reduction of the mutations have been observed in *Δsch9* cells and it was shown to depend on the

reduction of sensitivity to the superoxide-dependent DNA damage and the inactivation of the error-prone Rev1/Pol ζ polymerase that is involved in DNA-repair by translesion synthesis (TLS). [71].

Following the recent findings in the field of genomic instability, Sch9p is able to control the genomic instability in chronological ageing yeast by down regulating the expression of anti-oxidants genes such as *SOD2*, activating error-prone repair systems like Rev1/Pol ζ that generates mutations to repair oxidative DNA lesions when old cells resume growth, error-prone mitotic recombination and, finally, by regulating the production and catabolism of ethanol and acetic acid and the generation of carbon sources that reduce the ageing rate. [70, 71]

1.5.4. Chronological ageing and its relationship with Cancer

Yeast *SCH9* and *RAS2* are homologs of the mammalian proto-oncogenes Akt and Ras. In both yeast and mammals Ras and Sch9/Akt signal through pathways that regulate cell growth and promote ageing. In mammals, mutations to the oncogenes that activate Ras, Akt or in the upstream IGF-I-receptor are believed to promote cancer by allowing the survival and growth of cells that have accumulated mutations and that should be removed by apoptosis. Furthermore they have been proposed to promote genomic instability by allowing replication, promotion and increase of the damage in addition to the already present mutation. [77]

The accumulation of further mutations in cells that have already endured an initial oncogenic mutation is thought to be the responsible for tumor growth and metastasis.

2. Aim of the Thesis

The aim of the thesis is to define the stability of an induced translocated chromosome, during the Chronological Life Span of yeast, in comparison with a spontaneous translocation and to show which other possible gross chromosomal rearrangements may occur in a cell that has already endured a massive mutagenic event such as BIT. This would give us interesting insights on how the cell is able to adapt to new conditions and how a cell could recognize an unknown or remodeled chromosome.

3. Materials and Methods

3.1. Strains and Media

3.1.1. Wild type strains and Translocants

The diploid *S. cerevisiae* strain San1 was used for transformation with the kanamycin DNA cassette. It was obtained by mating Fas20 α , ade1, ade2, ade8, can1R, leu2, trp1, ura3-52 [78] and YPH250 a, ade2-101°, leu2- Δ 1, lys2-801a, his3- Δ 200, trp1- Δ 1, ura3-52 (ATCC 96519).

The translocant strains named D11, D3, D10 Big and D10 Small were generated by a translocation targeting chromosome XV on the *ADH1* gene and chromosome VIII on the *DUR3* gene. [52]

The translocant strains named Susu5, Susu7 and Susu9 were generated by a translocation targeting chromosome XVI on the *SSU1* gene and chromosome IX on the *SUC2* gene. In addition, these translocants have specific aneuploidies formed due to an adaptation to the initial BIT event. As shown in [54]

Finally the *rdh54 Δ /rdh54 Δ* and its respective translocants harbouring the same translocation as in D11, D3 and D10 were generated as explained in [53] and have an additional spontaneous translocation that occurred after the double knockout of *RDH54*. This translocation occurred between the an long terminal repeat (LTR) located near *DCD1* on chromosome VIII and a transposable yeast element YDRTy1-3 located on chromosome IV

All translocants strain harbour on the translocated chromosome the Kan^R for selection.

3.1.2. Media preparation

Media for Yeast was prepared following the standard protocols. YPD (Difco) supplemented with geneticin (G418) (final concentration 200 µg/ml, Gibco) was used as selective medium.

For the Chronological Life Span Assay synthetic complete media was used and prepared with ammonium glutamate instead than ammonium sulfate as previously described. [79]

The Drop-out mix used in the synthetic complete media was made as described in the table below:

Ingredients	mg. in 1000ml
Adenine	18
Arginine	76
Histidine	76
Leucine	380
Lysine	76
Methionine	76
Proline	76
Threonine	76
Tryptophan	76
Tyrosine	76
Uracil	76

Table 1: List of reagents used for the Drop-Out Mix

Tryptophan was added after autoclaving the media since it is thermolabile.

3.2. Chronological Life Span assay and Replica Plating

The chronological life span assay was performed by plating the strain from -80 on a YPD plate and left to grow for 4-5 days. Afterwards a single colony was picked and inoculated for a first overnight growth and then re-inoculated the day after to a

concentration of 10^5 cells/ml and left to grow at 30°C at 220rpm, this was counted as day 0 of the Life Span. Every 3 days a sample was retrieved and diluted accordingly to reach 300-400 Colony Forming Units (CFUs) per Plate. After the CFUs were counted the plate was replica-plated onto YPD + G418 200 µg/ml and left to grow for 1 day at 30°C. The colonies that are G418 sensitive were picked and analysed through Pulse Field Electrophoresis and Southern Blot to demonstrate the actual loss of the chromosome.

3.3. Pulse Field Electrophoresis and Southern Blot

Chromosomal separation was performed in a 1% pulse field certified agarose gel (BioRad) electrophoresis with the CHEF DR-II apparatus (BioRad) set at 200 V for 24h with an initial switching time of 60 s and a final time of 120 s. The agarose cell plugs were prepared following protocols described in Kaiser et al. [80]. Proteinase K (final concentration 20 mg/ml) used in the preparation of plugs was from Sigma. The fragmented DNA obtained by alkaline depurination was transferred by capillarity to a positively charged Hybond N+ nylon membrane (Amersham). Hybridizations were optimized from standard protocols [81] [52], and DNA detection was performed by using the CDP star ready-to-use chemiluminescent substrate (Roche). Probes were labeled using the polymerase chain reaction digoxigenin (PCR DIG) probe synthesis kit (Roche).

3.4. Table of Primers used for DIG labelled probes

The table below shows the list of primers used to produce the Digoxigenin Probes as specified by Roche. In addition the cartoon below shows the location where these probe hybridize on the translocated chromosome.

Name	Chromosome	Sequence
GAL4 fw	XVI	5' CCTCGAGAAGACCTTGACAT 3'
GAL4 rv		5' ATGGTCGGGAGCCTGTTAAC 3'
SGN1 Fw	IX	5' AAAGTGGATGCTAAGGCAAC 3'
SGN1 Rv		5' TGCCATTGTTGGAAATACTG 3'
CFD1 Fw		5' CAGACTGCGCTGACTCTTTG 3'
CFD1 Rv		5'TTGCAACACTCTGTGGGGTA
ALG6 Fw	XV	5' TAACTTCTGGTGC GTTACGA 3'
ALG6 Rv		5' AGGGCCAGGAGTTAAAGATT 3'
BRX1 Fw		5' GAAAAGAAGCAAGGCAATGT 3'
BRX1 rv		5' CCTCAGCAGCTTGTGTTTA 3'
OPI1 fw	VIII	5' ACGTCTCACAAGCTGACAAA 3'
OPI1 rv		5' AGTGGTCGTCCTAGCATTTTC 3'

Translocant IX-XVI



Translocant XV-VIII

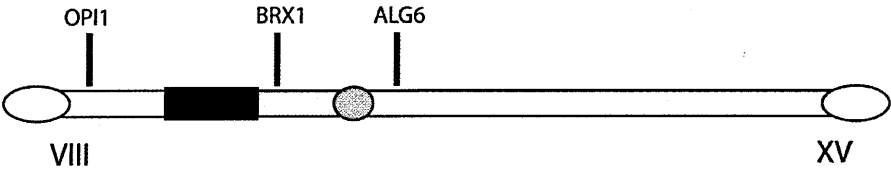


Figure 7: Cartoon showing where the probes anneal on the targeted chromosome. White ellipses represents the telomeres; Green dot represents the centromere; Blue, Purple, Yellow and Red represent respectively part of chromosome XVI, IX, VIII and XV. Black Bar represents the Kan^R. The figures are not to scale. Specific probes where amplified in order to target regions near the centromere and on the other wild type chromosome in order to have two references.

In addition this is the table of primers used for amplifying the DIG-probe that hybridizes near the centromere of chromosome V and VIII and the primers used for the amplification of the Ty elements located on chromosome V.

Name	Chromosome	Sequence
MNN1 fw	V	5' TTTCATCATAGAAGGCAGCA 3'
MNN1 rv		5' CGTTCAAGTCGGTTTTCTTT 3'
OSH7 fw	VIII	5' CACGGATGATATCGATGAGA 3'
OSH7 rv		5' TGTTCCTCCAAGAATGGGATT 3'
YERCTy1-1 Fw	V	5' CCAGGACATCCATAGCTTGT 3'
YERCTy1-1 Rv		5' CGCCTATGTACTTTCCACCT 3'
YERCTy1-2 Fw	V	5' AAGGACGGGTTTTGTCTCAT 3'
YERCTy1-2 Rv		5' GTTTGGGAACTGTTTCGAGG 3'

Table 2: List of primers used for probe amplification and for detection of Ty elements located on chromosome V

3.5. ROS Measurements

The cells, after they were grown overnight in order to make them reach plateau phase, were washed two times with PBS and resuspended in PBS with 5µg/ml of Dihydroethdium (DHE) and incubated for 15 min at 30°C in the dark under constant shaking. The fluorescence, having an excitation wavelength of 485nm and emission of 595nm, was measured in a BD Biosciences FACScalibur flow cytometer where 100,000 cells were counted for each sample. This method is very sensitive for ROS measurements and it is given by the oxidation of DHE into ethidium and 2-hydroxy ethidium which both give fluorescence. [82, 83] In addition, cells were also picked at their late CLS, ranging from 20%-10% survival of the population, and their ROS levels were measured as mentioned above. A control staining was done with

Propidium Iodide (PI) by incubating them in PBS with 10 µg/ml of PI and counting them by FACS. [84] Positive PI staining indicates permeabilized (dead) cells.

3.6. Genomic Extraction and Gene Copy Number qPCR

Genomic DNA was extracted from an entire yeast colony, which was left to grow for at least 3 days, using the Wizard Genomic DNA Purification Kit from Promega with the protocol provided. Afterwards the DNA was quantified at the GeneQuantpro spectrophotometer and diluted in order to have serial dilutions of 10 to 50 ng/µl of genomic DNA.

For the Gene Copy Number qPCR the DNA was diluted in order to design a standard curve. Reference genes *ACT1* on chromosome VI and *SSE2* on chromosome II were used to design the standard curve being housekeeping and haplo-insufficient genes. The Kan^R gene was used to quantify the gene copy number of the translocated chromosomes in both G418 resistant colonies (control) and G418 sensitive colonies. The kit used for the qPCR is the Rotor-Gene SYBR green KIT (Qiagen) with the already provided PCR program designed for the Rotor-GENE Q Real Time PCR from Qiagen.

The formula used for the calculation of the gene copy number follows the Absolute Quantification method where a standard curve is designed with a known amount of DNA and a known gene copy number and is compared to an unknown gene copy number. The formula of the standard line is: $Y = -1,05 * X + 10,067$

The table below shows the primers used for the qPCR, they are desgined in order to have a GC amount between 40% and 60% and to produce an amplicon not larger than 200 base pairs as specified by the Kit.

Name	Chromosome	Sequence
ACT1- qPCR-Fw 2	VI (housekeeping)	5'-GGTAGACCAAGACACCAAGG-3'
ACT1-qPCR-Rv 2		5'-TTTTCCATATCGTCCCAGTT-3'
SSE2-qPCR-Fw	II (housekeeping)	5'-GCTTTCATATGTGCCATTCA-3'
SSE2-qPCR-Rv		5'-TGCTGAAATCTCCAGTACGA-3'
KanR-qPCR-Fw	translocated	5'-ATAATGTCGGGCAATCAG-3'
KanR-qPCR-Rv		5'-GAGGCATAAATTCGTCA-3'

Table 3: Pimers used for the Gene Copy Number qPCR. Translocated refers to the translocated chromosome.

3.7. Annexin V staining

The Annexin V-FITC staining was performed by initially removing from the Chronological ageing cells the cell wall by washing them in Sorbitol Buffer (1.2M sorbitol, 0.5 mM MgCl2, 35 mM potassium phosphate, pH 6.8) and then incubating them for 1 hour in Sorbitol Buffer with 5.5% glusulase (Roche) and 15 U/ml lyticase (Sigma Aldrich) in order to expose the phosphatidylserine. Afterwards the cells were washed with Incubation Buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2 containing 1.2M Sorbitol) and incubated for 20 minutes in 20µl of Incubation Buffer/Sorbitol with 2µl of Annexin V-FITC (ready to use from Roche) and 2µl of Propidium Iodide 500µg/ml (ready to use from Roche). The cells were harvested and analyzed with a FACScalibur BD biosciences flow cytometer counting 30,000 cell per sample and photographed with an Inverted Fluorescent Microscope Fluorvert

from Leitz with the correct filters for the fluorescence visualization. This method discriminates apoptotic cells from necrotic cells [85, 86]. Cells with positive staining for Annexin and PI are necrotic (already dead) while cells positive for Annexin but negative for PI are in an early stage of apoptosis.

3.8. Bioinformatics analysis

The Bioinformatics analysis of the Genetic and Physical interactions of the Genes was performed using the GeneMANIA algorithm server [87] also suggested on the *Saccharomyces Genome Database* (SGD). The genes analysed were mainly oxidative stress regulators and mitochondrial homeostasis genes that were retrieved from a list of approximately 1000 genes found to have more than 2 fold gene expression deregulation after a high throughput MicroArray Gene Expression Analysis done on the strain D10-Big in the paper (Nikitin et al. In production).

4. Results

4.1. Different Chronological Life Spans arise from the same Bridge Induced Translocations

As previously described two different sets of translocation were analysed, one having the translocation between chromosomes XVI (*SSU1*) and IX (*SUC2*) [54] and one having the translocation between chromosomes XV (*ADH1*) and VIII (*DUR3*) [52]. The chronological life span displayed different outcome for each strain deriving from the same translocation. For the translocation XVI-IX, Susu5, Susu7 and Susu9 were taken into consideration. Susu5 exhibited a very short life span and a mildly short lifespan was found for Susu7 (Figure 8). The very short life span of Susu5 is most probably due to its inability to grow on non-fermentable carbon sources (Nikitin et al. in production) such as glycerol or acetate and therefore, once the glucose is depleted in the synthetic media, it is not able to respire in stationary phase and dies quickly. In whole genome high throughput investigations of CLS it was found that non-respiring strains generally have a short lifespan [88, 89]. Even more interesting is Susu9 that has adapted to the translocation enhancing its life span. This different variety of life spans is most probably due to the high gene deregulations that occur after translocation as shown by the paper of Dr. Nikitin [55] and shows how every translocant adapts differently to the same BIT.

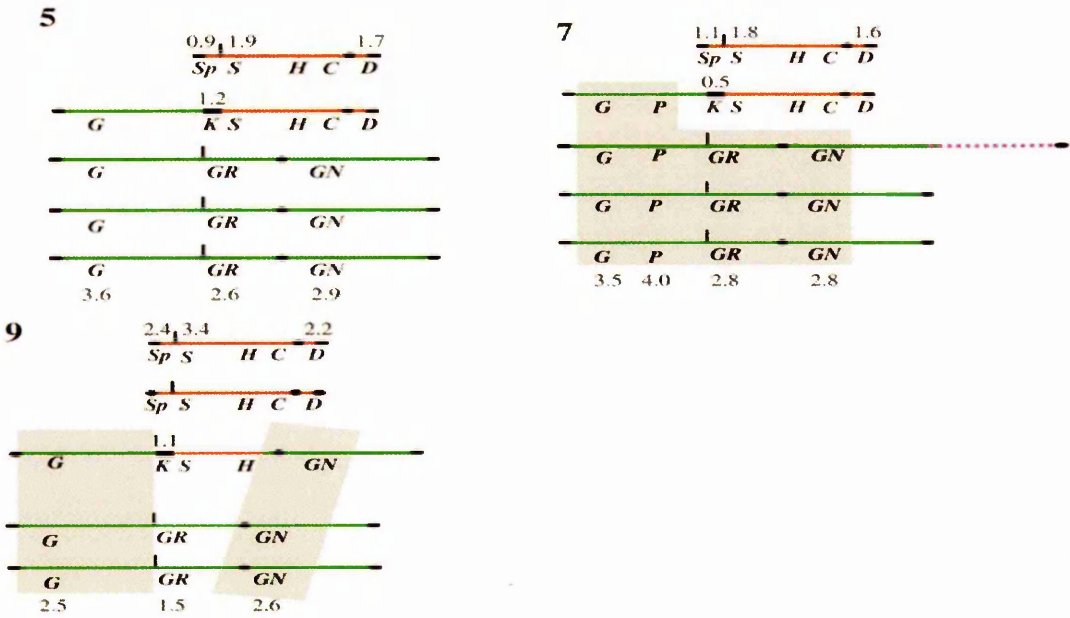
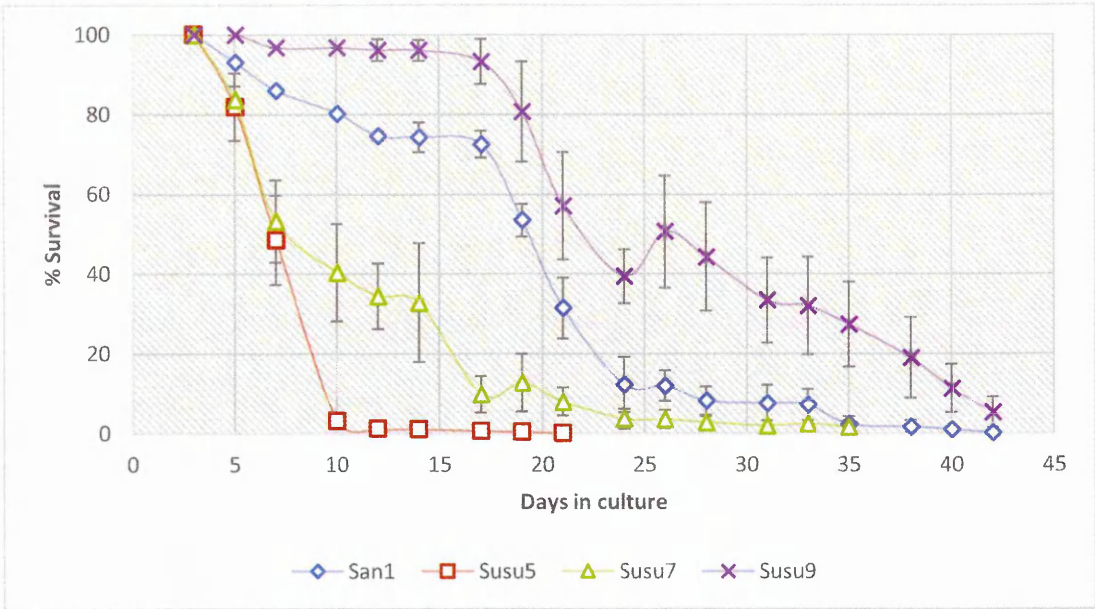


Figure 8: Upper panel graphic representation of the CLS of the translocants SUSU5 (red square), Susu7 (green triangle), Susu9 (purple cross) compared to wild type San1 (blue diamonds). The percentage of survival (Y axis) was normalized on day three to be 100%. This graph clearly shows the very different life span outcomes of the translocants after the BIT event between chromosome XVI targeting *SSU1* and chromosome IX targeting *SUC2*. Black bars represent the standard error. Lower panel schematic representation of the different aneuploidies of the translocant strains. The figure was adapted from [54]. Gene copy-number values are also indicated. G, GAL4; GR, GLR1; GN, GLN1; S, SUC2; Sp, SUC2 promoter; H, HIS5; C, CFN1; D, DAL4; K, KANMX4. Numbers 5, 7, 9 indicate Susu5, Susu7 and Susu9

All the life span differences between the translocants and the wild type San1 are statistically significant since the p value calculated with the Student T-Test is well below 0.001.

The other sets of translocants (Figure 9) harboring a translocation between chromosomes XV targeting *ADH1* and VIII targeting *DUR3* also show a very different outcome of life span but with D11 and D3 having a wild type-like lifespan and D10-big and D10 small exhibiting a shorter life span. Again the short life span of these translocants is most probably due to their inability to grow on non-fermentable carbon sources. (Nikitin et al. in production) It is important to note that these translocants didn't form any aneuploidy after the initial BIT event.

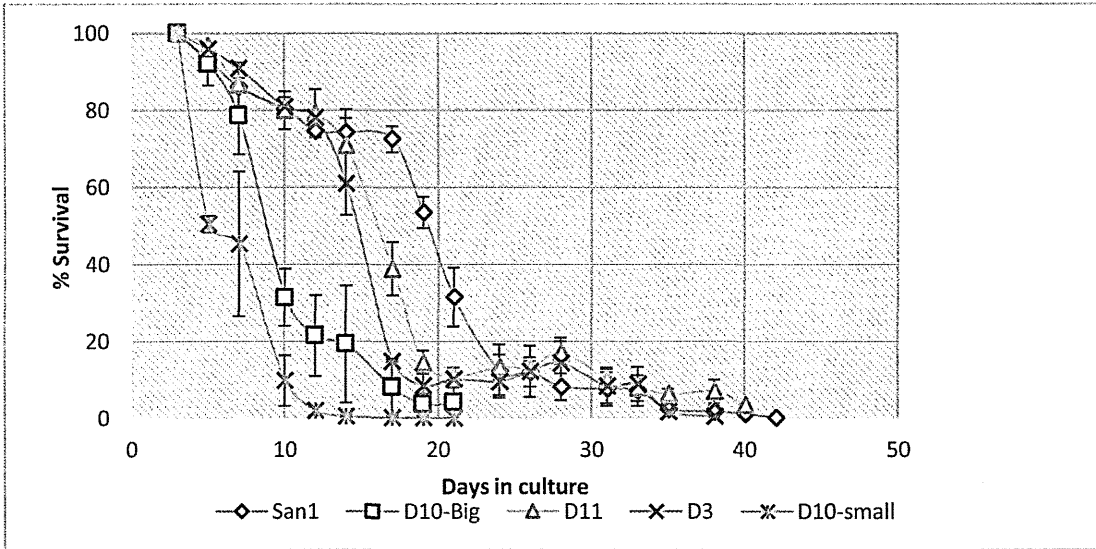


Figure 9: Graphic representation of the CLS of the translocants D10-Big (red square), D11 (green triangle), D3 (purple cross), D10-Small (light blue cross) compared to wild type San1 (blue diamonds). The percentage of survival (Y axis) was normalized on day three to be 100%. This graph clearly shows the very different life span outcomes of the translocants after the BIT event between chromosome XV targeting *ADH1* and chromosome VIII targeting *DUR3*. Black bars represent the standard error.

The last and third group of translocants have the same translocation as the one previously described for the strains D11, D3, D10-big and D10-small but are based on the wild type San1 with a *rdh54Δ/rdh54Δ* background [53].

In these particular strains named RDH54-Del for the wild type and Clone 4, Clone 7 and Clone 16 for the bridge induced translocants derived from RDH54-Del, because of the lack of *RDH54*, the loss of the translocated chromosome was enhanced. Furthermore RDH54-Del had an autonomously induced translocation, between chromosome IV and VIII, the formed after the double knock-out of *RDH54* and this spontaneous translocation was maintained after the bridge induced translocation [53]. Therefore, the strains Clone 4, Clone 7 and Clone 16 have a spontaneous translocation together with an induced translocation. This gave the opportunity to see if the yeast is able to discriminate between a spontaneous vs. an induced translocation and to see if the genomic stability decreases in the absence of *RDH54*. As shown in Figure 10 the life span of these translocants is wild type-like with the only exception of Clone 16 that has a mildly shorter life span.

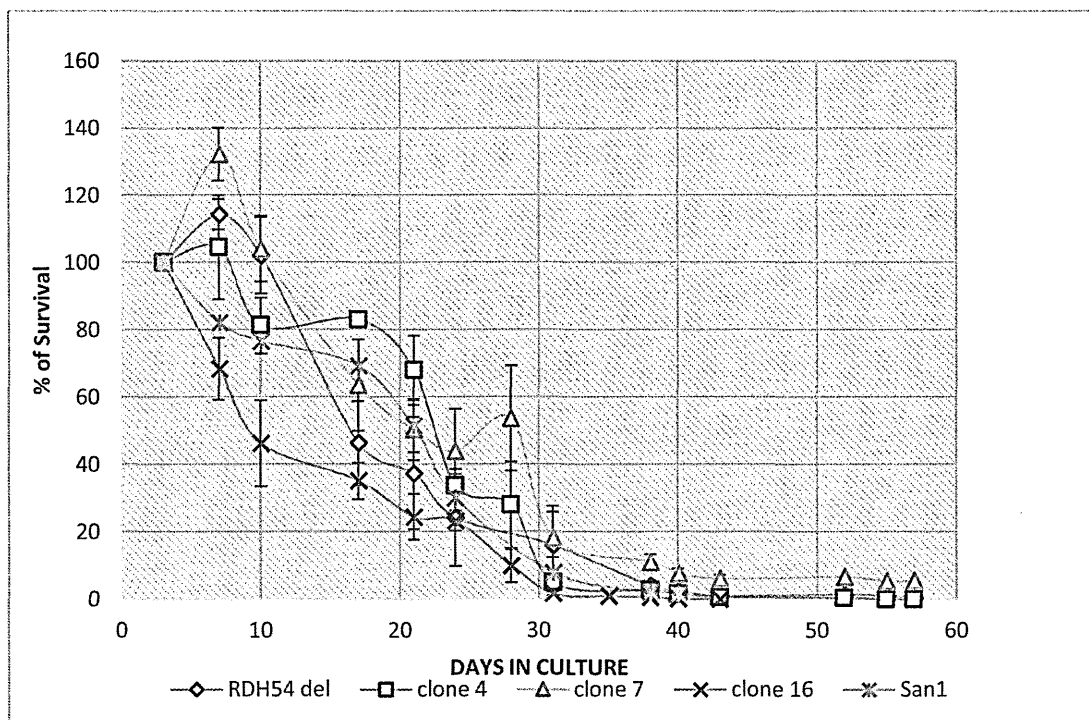


Figure 10: Upper Panel Graphic representation of the CLS of the translocants Clone 4 (red square), Clone 7 (green triangle), Clone 16 (purple cross) compared to wild type San1 (light blue cross) and RDH54-del (blue diamonds). The percentage of survival (Y axis) was normalized on day three to be 100%. This graph clearly shows the similar life span outcomes of the translocants after the BIT event between chromosome XV targeting *ADH1* and chromosome VIII targeting *DUR3*. Black bars represent the standard error. Lower panel, schematic representation of the spontaneous translocation present in all RDH54 double knockout backgrounds. Black asterisk marks the exact point of translocation. The figure was adapted from [53]

This indicates that the absence of *RDH54* and the spontaneous translocation did not influence the CLS of these translocants

4.2. Reactive Oxygen Species levels of the translocants are not related to their life span and dramatically increase with the age of the population.

Reactive Oxygen Species (ROS) levels deeply influence the chronological life span of yeast by inducing them to apoptosis [86] and are usually negatively related to the CLS. This situation was not presented when the translocant strains were analyzed with DHE staining, in fact the result was an extremely high increase of ROS levels at 20% of survival (Figure 11). In addition Susu7 showed a higher level of ROS already at 100% of survival. It was even more interesting to note that if we refer to their life spans, most of the translocants have wild type like CLS or in the case of Susu9 longer CLS. This indicates that the translocants have adapted to the extremely high ROS levels so well that one of them was even able to have a longer life span.

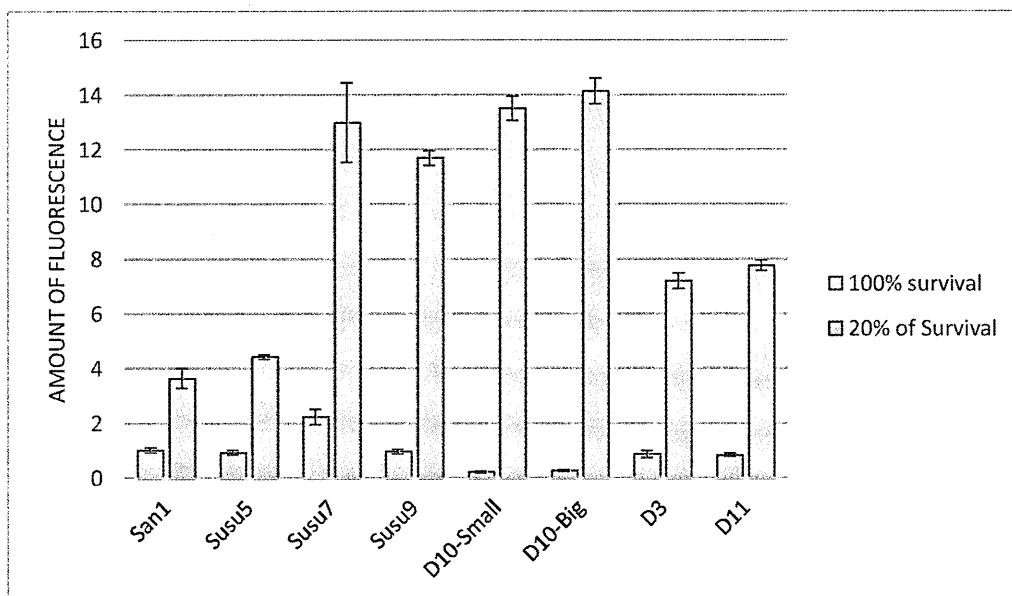


Figure 11: ROS levels of the translocants and the wild type San1 measured by Flow Cytometry after DHE staining. All the values referring to the amount of fluorescence were normalized to the value at 100% survival of San1 that was counted as 1. All the results are statistically significant having a p value, calculated using the student T-test and comparing the amount of fluorescence of the translocants with the wild type, less than 0.01. Black bars represent the standard error.

But the DHE staining could show false positive results since it could stain also necrotic cells that in reality are not producing ROS anymore. A control stain was done with Propidium Iodide that enters damaged membranes hence necrotic cells, just to discriminate the false positives with the actual increase in ROS.

Figure 12 elucidates the comparison between the PI staining and the DHE staining, showing that the increase in ROS is not due to necrotic cells staining.

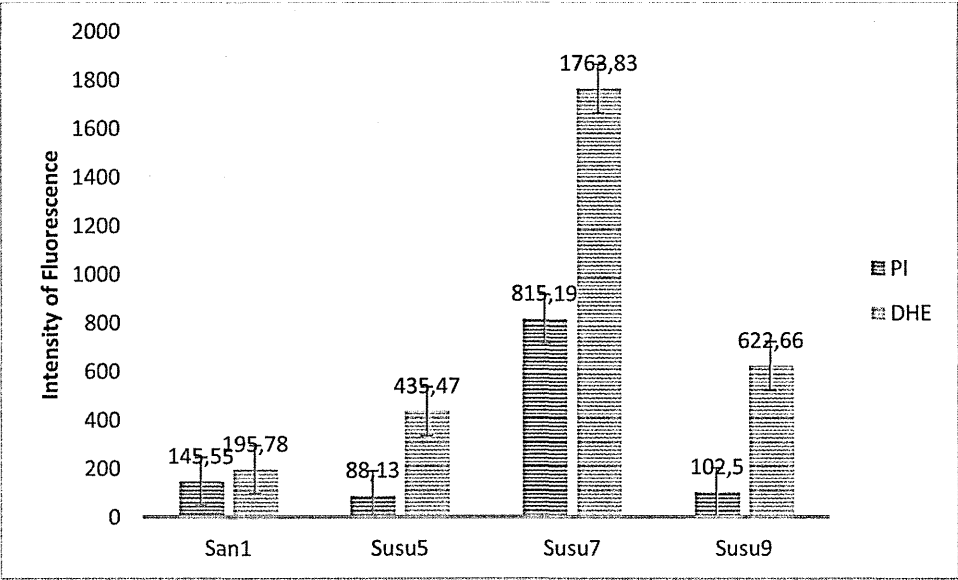


Figure 12: Propidium Iodide staining of San1, Susu5, Susu7 and Susu9 compared to the DHE staining. As shown by the graph the DHE staining actually represents an increase in ROS and is not biased by the staining of Necrotic Cells. All the experiments were performed three times with similar results with a p value below 0,01.

The same results were also shown by measuring the ROS levels of the *rdh54Δ/rdh54Δ* (RDH54-del) and its respective translocants (Figure 13). Furthermore since the *RDH54* double knockout wild type has a spontaneous translocated chromosome as demonstrated by [53], it also shows a very high

increase in ROS levels confirming that the ROS increase is not only due to an induced translocation but to translocations in general.

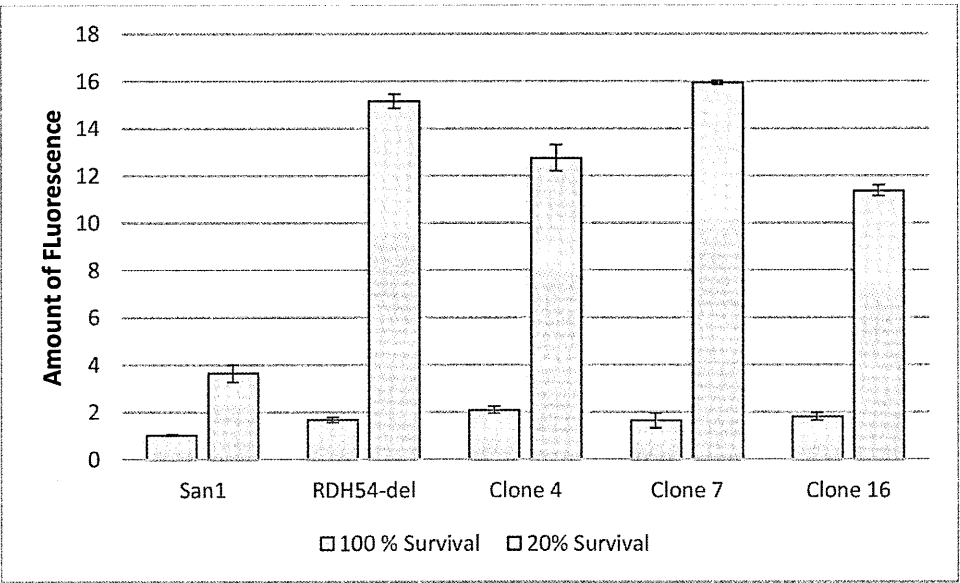


Figure 13: ROS levels of San1, RDH54-del and its respective translocants (Clone 4, Clone 7, Clone 16). The dramatic increase in ROS levels is clear not only for the induced translocation but also for the spontaneous one that has formed after the initial knockout of *RDH54* in San1 as shown by RDH54-del. Black Bars represent the standard error and the p values of all the strains compared to the wild type San1 have a value below 0.01.

If we compare the ROS levels with the life span of Clone4, Clone7, Clone 16 and RDH54-del, we can see that they all have a wild type like life span indicating that also in this case translocant ROS levels do not correlate with the life span and most probably translocant cells have adapted to a very high level of oxydative stress.

4.3. Translocant cells exhibit higher apoptosis level at the beginning of their Chronological Life Span regardless of their ROS levels

Due to their abnormal ROS levels during their life span it was interesting to see if the translocants exhibit the apoptotic markers as they should during their chronological life span. Annexin V and PI staining were performed at their initial and mid-life span in order to discriminate between apoptotic and necrotic cells. The results show that the translocants at the beginning of their life span (Day1) have a higher level of apoptosis if compared to the wild type (Figure 14), which is in accordance with the previous paper of Dr. Rossi [54] where it was shown that the translocants have a higher death rate at late-mid log phase. At day 6, though, Susu9 has a mildly increase of apoptosis from its initial Day 1 whereas Susu5 and Susu7 have a dramatic increase in cell death in accordance with their CLS, with Susu5 having preferentially necrotic cells (Figure 15). This could be due to its respiratory deficiency.

These results give further indication that even if the cells exhibit high ROS levels their CLS and Apoptotic pathway have not been influenced demonstrating that these strains have adapted very well to the high ROS levels.

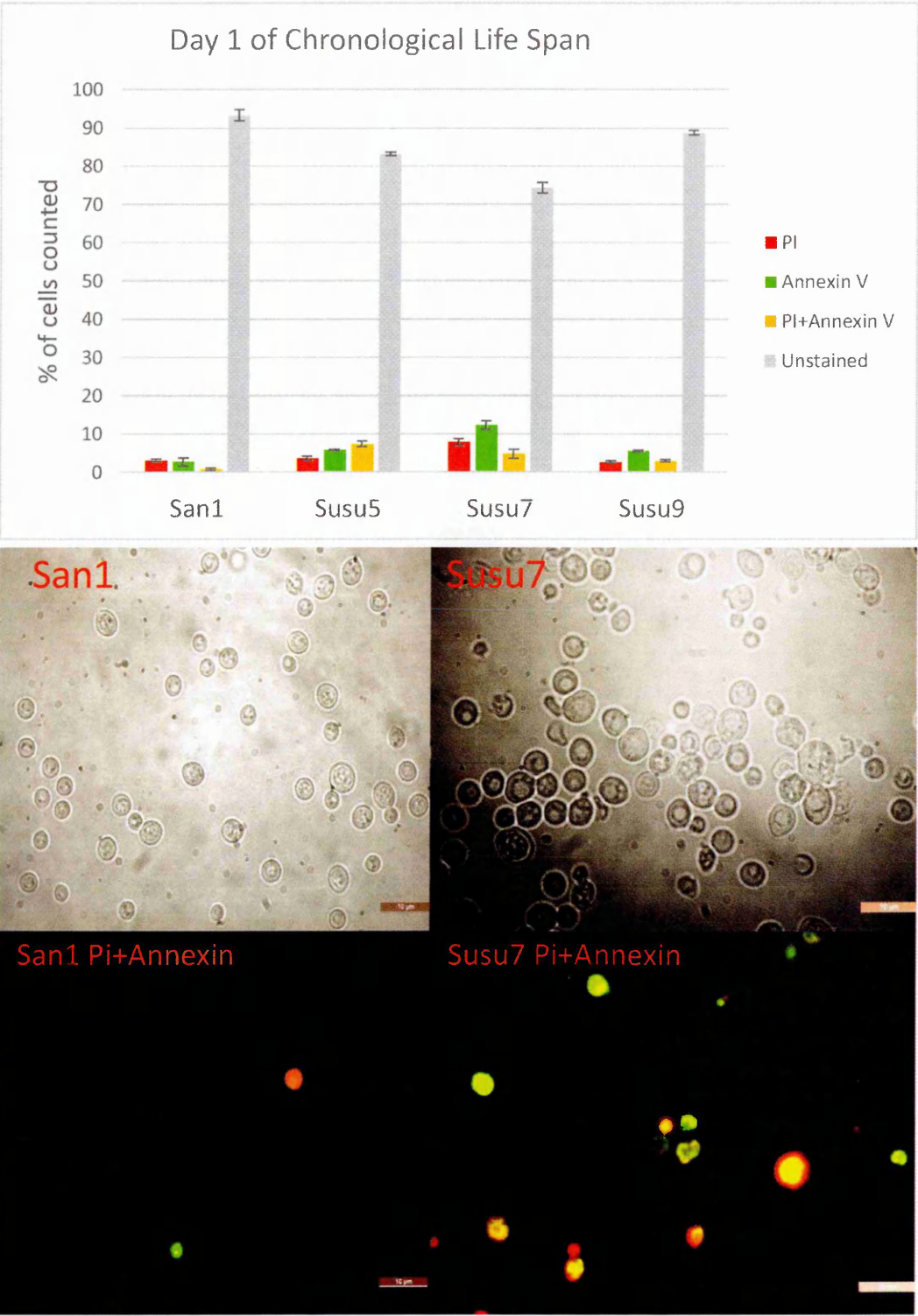


Figure 14: At the top, the graph shows the percentage of cells counted by flow cytometry at day 1 of CLS being Annexin V positive (green), Propidium Iodide positive (red), Propidium Iodide and Annexin V positive (yellow) and unstained (light Grey). Black bars show the Standard Error. At the bottom two pictures of San1 and Susu7 at day1 unstained and stained with Propidium Iodide + Annexin V. The pictures were taken at a magnitude of 100X. Brown bars indicate the scale of 10µm.

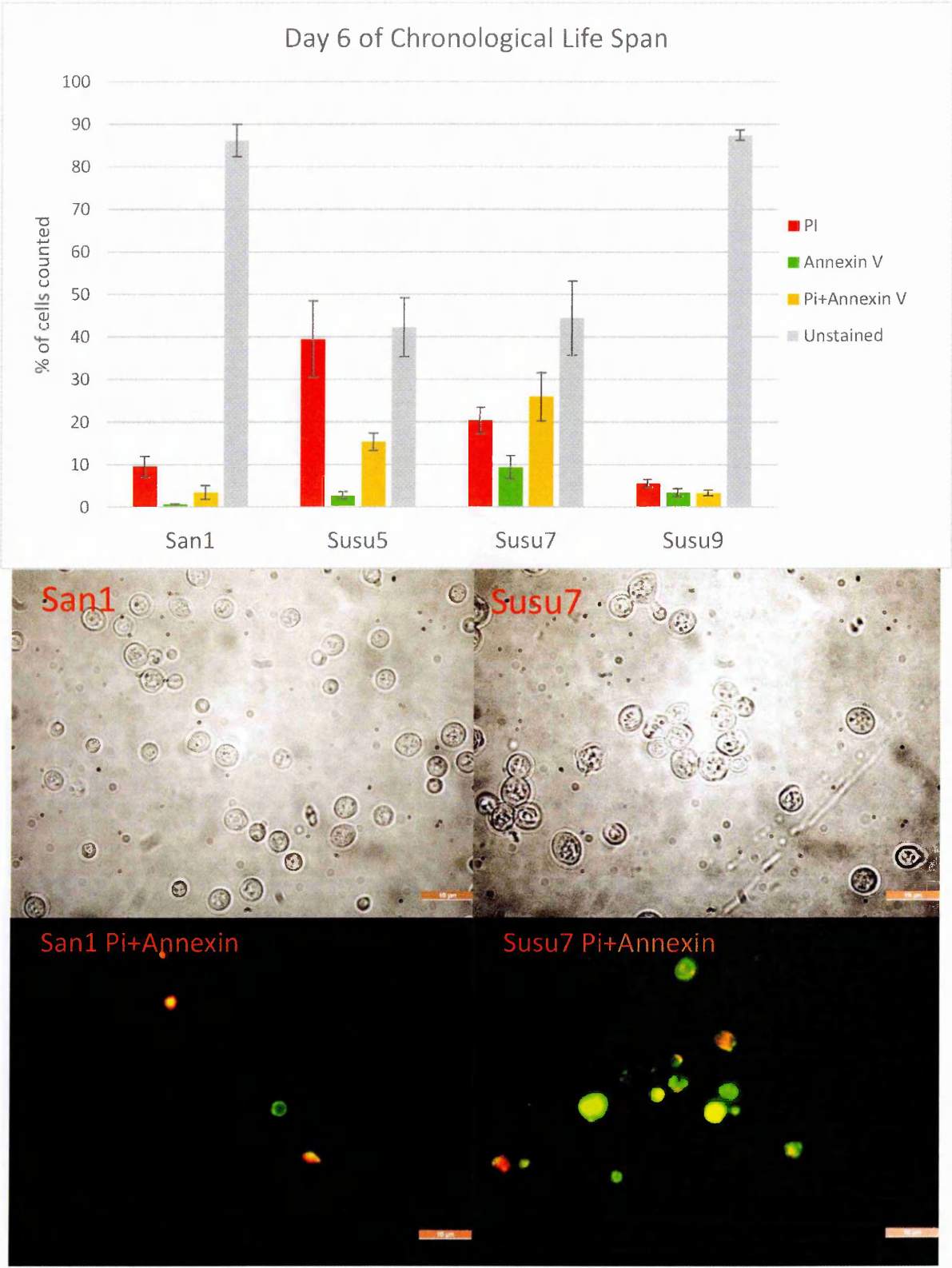


Figure 15: At the top, the graph shows the percentage of cells counted by flow cytometry at day 6 of CLS being Annexin V positive (green), Propidium iodide positive (red), Propidium iodide and Annexin V positive (yellow) and unstained (light Grey). Black bars show the Standard Error. At the bottom two pictures of San1 and Susu7 at day6 unstained and stained with Propidium iodide + Annexin V. The pictures were taken at a magnitude of 100X. Brown bars indicate the scale of 10µm.

4.4. Loss of the translocated chromosome is loci-dependent and is preferred to the spontaneous translocation.

Now that the Chronological Life Span has been characterized with the ROS levels of every translocant, the stability of the translocated chromosome must be evaluated. In order to initially screen the instability of the TrChromosome every Colony Forming Unit (CFU) counted for the determination of the CLS was replica plated on YPD+G418 plates thereby determining the loss of the Kan^R marker, located on the Translocated Chromosome (TrChromosome), by counting the G418 sensitive colonies.

The translocants D3, D10-Big and D10-Small show no loss of the resistance marker at all, whereas D11 shows a low level of loss that reaches its peak at 2% at the end of the life span (Figure 16)

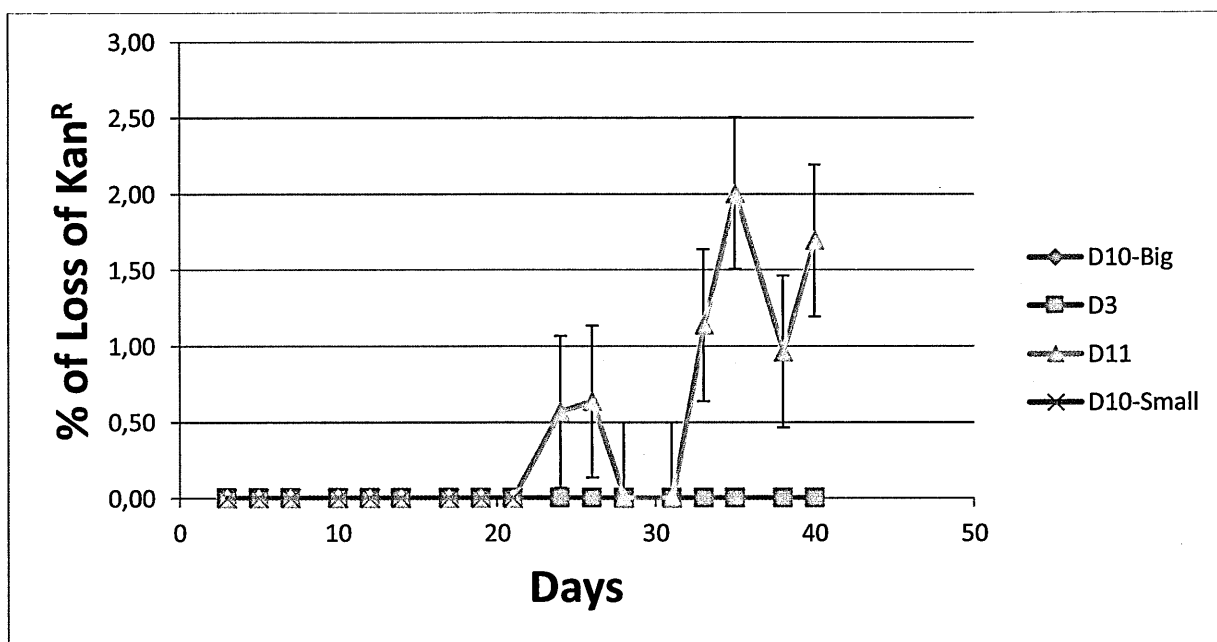
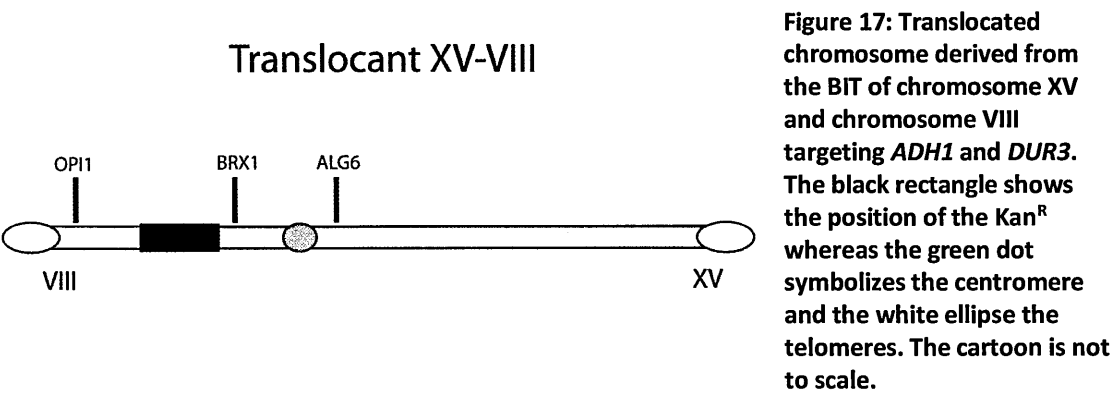


Figure 16: Percentage of G418 sensitive colonies during the chronological ageing of the translocants D10-Big, D11 and D3. D11 is the only strain that shows a loss of the resistance with a maximum of 2%. For every time point approximately 1000 colonies were replicated and the percentages calculated accordingly.

In order to determine if this was actually a loss of the chromosome, all the colonies that were G418 sensitive were picked and analyzed by Pulse Field Electrophoresis and afterwards Southern Blotted probing near the centromere. This would demonstrate that with the lack of the centromere the chromosome cannot be segregated during mitosis therefore lost or rearranged. Figure 17 is a schematic representation of where the chromosome was probed in order to define the loss of the chromosome.



The pulsed field electrophoresis (Figure 18) shows that all the chromosomes of the G148 sensitive colonies are present at the correct position and that no major rearrangement has occurred within the genome.

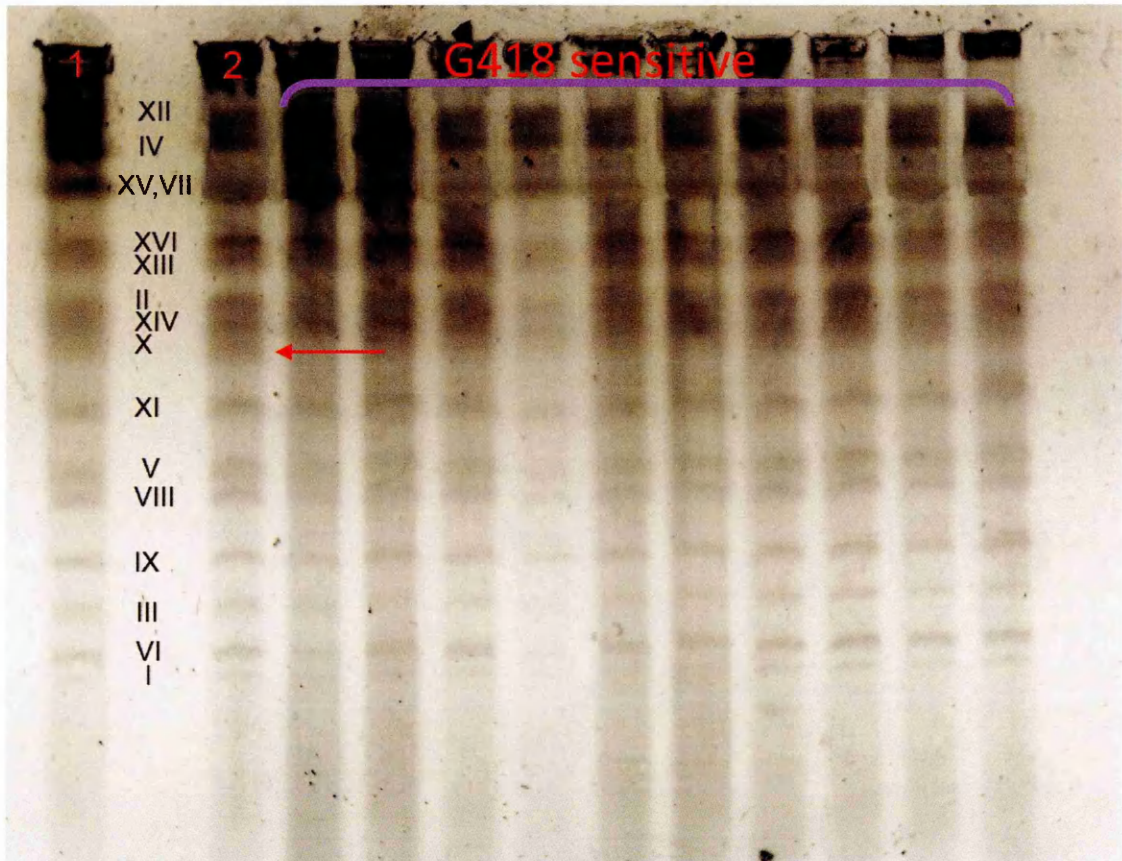


Figure 18: Pulse Field Electrophoresis gel of wild type San1 (lane 1), D11 at 100% of survival (lane 2) and chronologically aged D11 sensitive to G418. The Roman numbers represent the height of the respective chromosomes. The Translocated Chromosome is indicated with a red arrow.

But the Southern blot shows clearly that the translocated chromosome was lost since no signal of the probes located near the centromere (*BRX1* and *ALG6*) were detected for the G418 sensitive colonies whereas for D11 at 100% of survival there is a clear band (Figure 19). In addition some colonies have rearranged the *BRX1* locus in other chromosomes that were not identified. This is evidence that most probably the chromosome is not entirely lost but that some loci are kept and rearranged with other chromosomes. The fact that just the strain D11 loses the chromosome could be partially explained by the fact that D11 has a shorter TrChromosome that had a rearrangement [52].



Figure 19: Southern Blots of the Pulsed Field Gel showed in Figure 18. Panel A shows the probing of chromosome VIII on the OPI1 locus; lane 1 is the wild type San1 with the wild type chromosome VIII hybridized; lane 2 is D11 at 100% of survival with hybridized both wild type chromosome VIII and translocated chromosome; purple parenthesis comprehends the G418 sensitive colonies of aged D11 that have no signal for the translocated chromosome. Panel B and C are the same as Panel A just that the regions probed are BRX1 and ALG6 hybridizing chromosome XV and the translocated chromosome and that the blue arrows on panel B show the rearrangement of the BRX1 locus for two G418 sensitive colonies.

The translocation XVI-IX targeting *SSU1* and *SUC2* is much more unstable (Figure 20) in fact as shown by the initial screening for G418 sensitive colonies all of the translocant strains (Susu5, Susu7, and Susu9) lose at different percentages the translocated chromosomes. An explanation could be that the stability is also partially due to the loci targeted for translocation and most probably due to the rearrangements that occur in order to balance the different translocations. In fact all these translocants have different aneuploidies with Susu9 having a longer induced TrChromosome which is more stable.

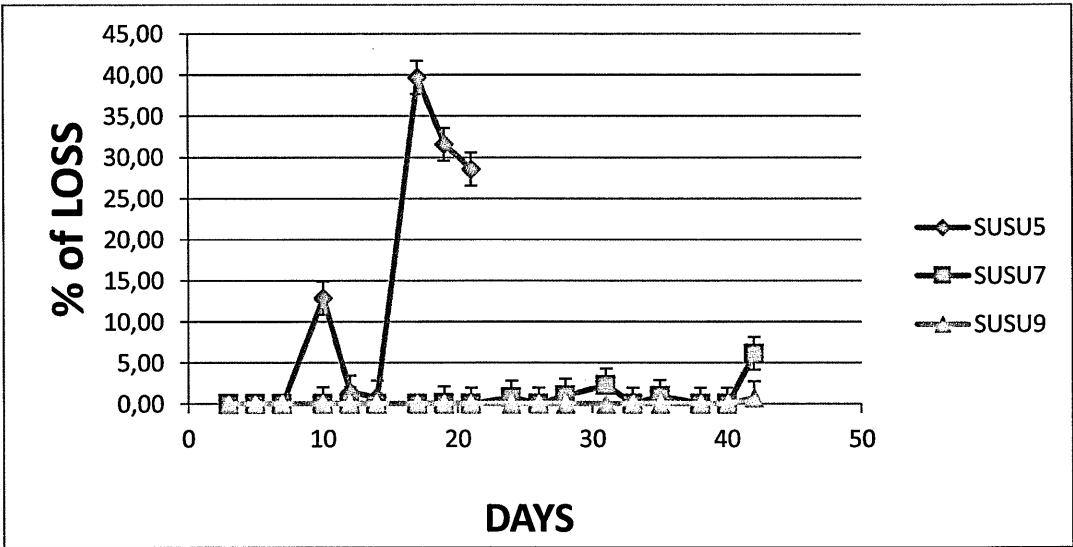


Figure 20: Percentage of loss of the Kan^R after replica plating. Susu5 shows a maximum loss of 40%, Susu7 shows a maximum loss of 5% and Susu9 shows a maximum loss of 1%. For every time point approximately 1000 colonies were replicated and the percentages calculated accordingly.

The same procedure followed for D11 was also done for the Susu translocants, in order to determine if this was an actual loss of the translocated chromosome. The TrChromosome was hybridized near the centromere with probes SGN1 and CFD1 and with GAL4 for second reference which also probed the spontaneous rearrangement that occurred after BIT in Susu7 [54]. All of the Susu translocants showed a loss of the chromosome since there was no signal when probing near the centromere. In addition Susu7 showed some anomalies both on Pulse Field Electrophoresis gel and after hybridization. As shown in Figure 21, in two samples of aged G418 sensitive Susu7 colonies the bands of Chromosome VIII and V merged to give a single band, and the same was true for chromosome XVI and XIII.

This is most probably due to a Transposable Yeast Element (Ty) rearrangement since after probing near the centromere for chromosomes V and VIII both gave a positive signal but when analyzing the presence of the Ty elements located on chromosome V by PCR no amplification was seen on the electrophoresis gel (Figure

These results demonstrate that Susu7 has a very unstable genome with a good contribution presumably given by the high levels of ROS generated already at the beginning of its life span. Furthermore, in accordance with the results of Susu7, where most of the natural aneuploidies were kept rather than the translocated chromosome also Susu5 kept the rearranged chromosome but lost the induced TrChromosome (Figure 23).

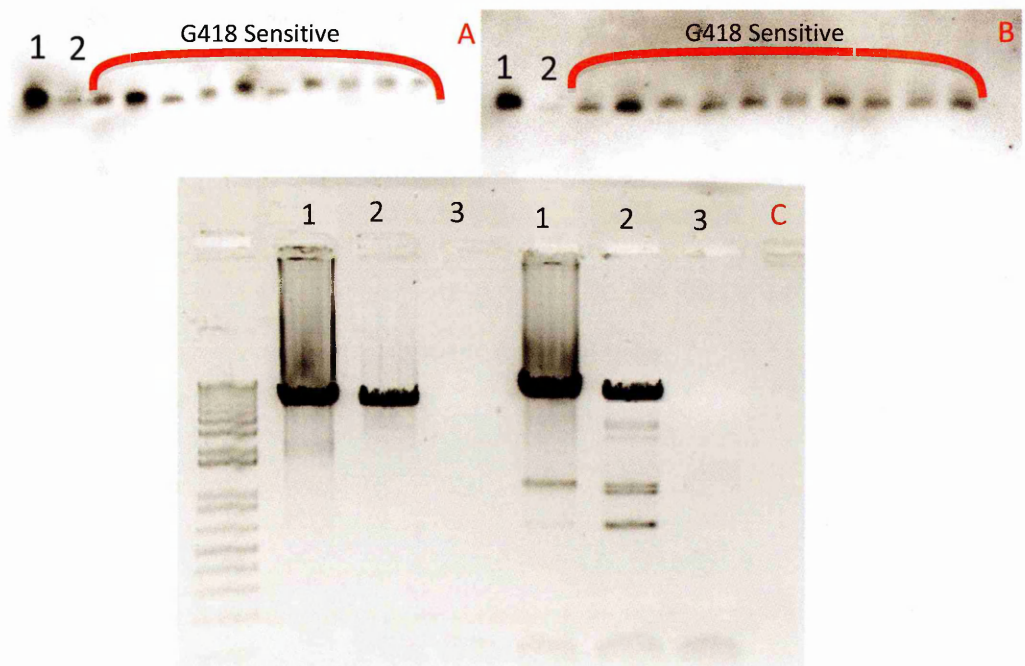


Figure 22: Panel A and B: Southern Blot hybridizing near the centromere of chromosome V and VIII respectively. Lane 1 is San1 wild type, Lane 2 is Susu5 at 100% of survival and all the others are aged Susu5 G418 sensitive colonies. Panel C: is an electrophoresis gel showing the amplification of the Ty element: in lane 1 San1 wild type, in lane 2 Susu5 at 100% survival and in lane 3 the colony with the merged chromosome V. This results suggests that both chromosomes are present but merged at the same height since some genetic information was most probably rearranged. The primers used for the amplification of the Ty are described in the M&M.

The results in this paragraph show that the instability of TrChromosome is loci dependent since it is much more unstable in the translocation between chromosomes XVI and IX rather than the translocation between chromosomes XV

and VIII. In addition it was proven that cells prefer to retain the spontaneous abnormal chromosome, since it is formed due an adaptation, in contrast to the induced TrChromosome. In fact Susu5 retains the spontaneous rearrangement even if it is much shorter in length than the induced TrChromosome.

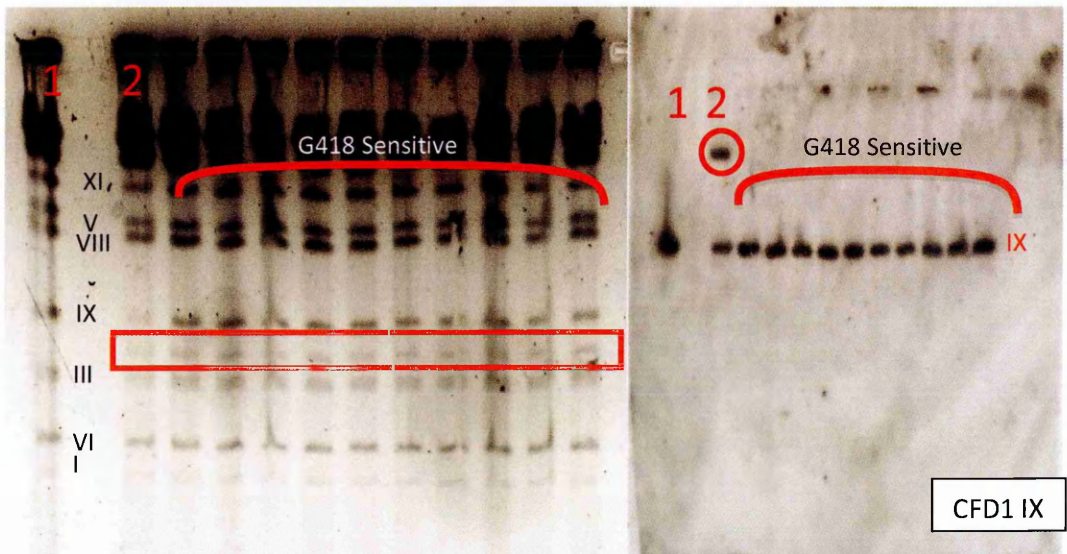


Figure 23: On the left a pulse field electrophoresis gel run with the program to separate short chromosomes. On the right Southern Blot hybridization performed on the gel on the right probing chromosome IX. Lane 1 represents wild type San1 and Lane 2 represents Susu5 at 100% of survival, all the others are aged Susu5 G418 sensitive. Red rectangle shows the spontaneous rearrangement present in all of the G418 sensitive colonies and in Susu5 at 100% of survival. Red Circle indicates the presence of the translocated chromosome.

4.5. *rdh54Δ/rdh54Δ* translocants have a steady increase of loss of the TrChromosome.

Previously it was proven that translocants with an *rdh54Δ/rdh54Δ* background have a loss of the TrChromosome already at the beginning of their life span as shown by [53], and this is associated most probably to the fact that *RDH54* has a role in pairing the chromosomes during mitosis [91].

It was of interest to analyze if there could be an increased loss of the chromosome with the increasing of the age of the population for the very stable translocation

between chromosomes XV and VIII. Therefore, the procedure to prove the concept was the same used for all the other translocants, with an initial replica plating on G418 to screen the loss of the Kan^R and, afterwards, a Pulse Field Electrophoresis with a subsequent Southern Blot probing near the centromere of the translocant chromosomes to actually evaluate the loss.

The results suggest that the translocants have an increasing level of loss of the TrChromosome with age (Figure 24). This exponential level of loss is very well demonstrated by Clone 4 that starts with an initial loss of 4% at day 3 of ageing and ends with a loss of more than 20% at day 30. In addition it was interesting to find that all the G418-sensitive colonies retained the spontaneous translocated chromosome, even though shorter in length than the induced TrChromosome, formed after the KO of *RDH54* in contrast to the induced TrChromosome. (Figure 25)

These results suggest that *RDH54* may play a role in the stability of the induced TrChromosome also during ageing and that the cells preferentially lose a translocation that's been induced in contrast to one that has formed spontaneously due to an adaptation.

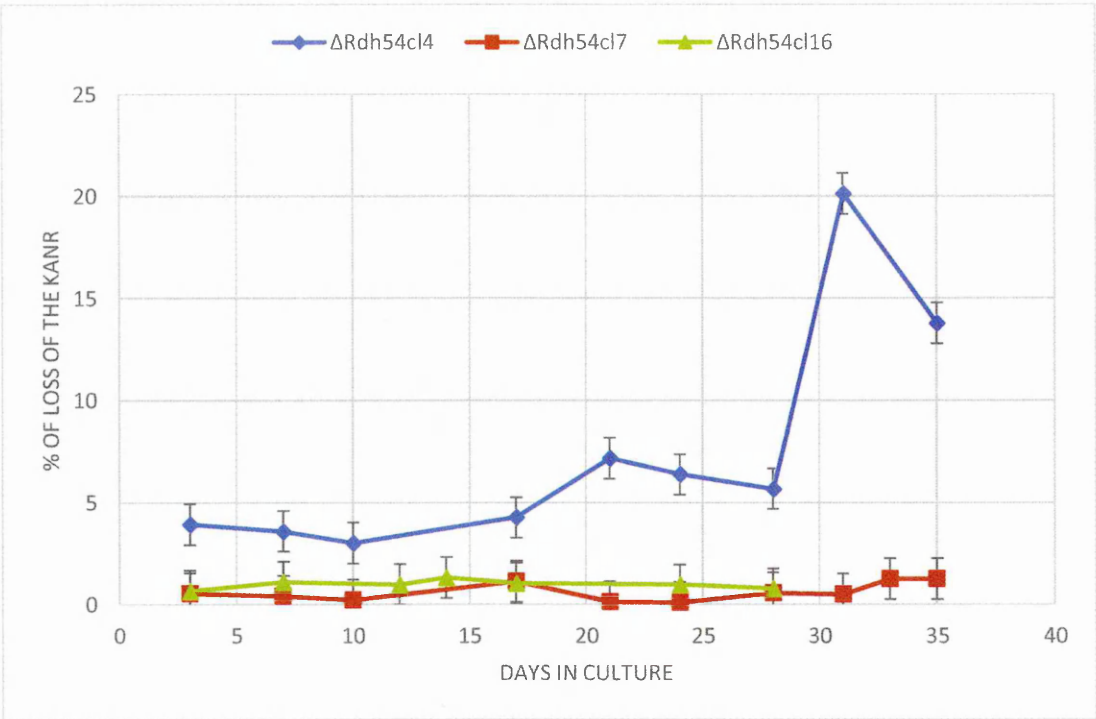


Figure 24: Percentage of G418 sensitive colonies after replica plating. The increase in the loss of the chromosome is very clear and significant for Clone 4 (Blue Diamond) and a bit less clear but still reliable for Clone 7 (Red Square) and Clone 16 (Green Triangle). For every time point approximately 1000 colonies were replicated and the percentages calculated accordingly.

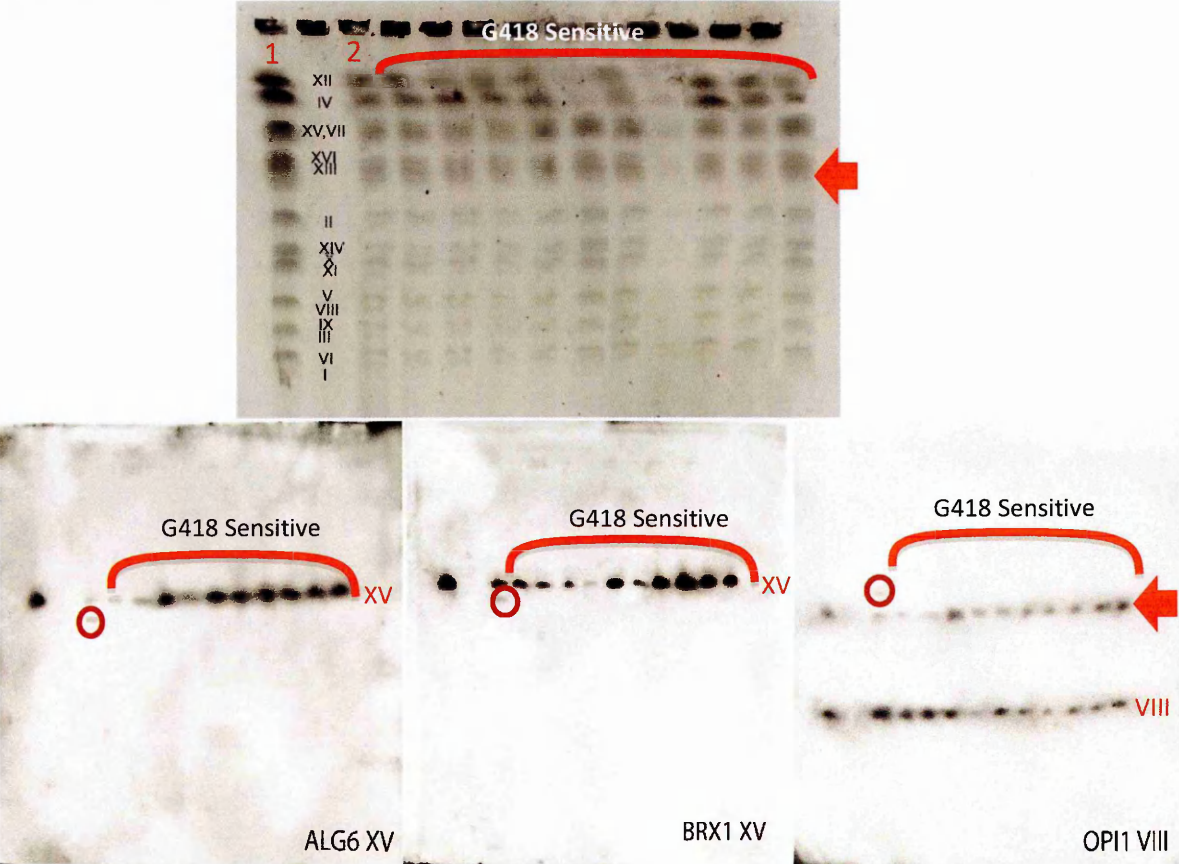


Figure 25 Pulse field electrophoresis gel of RDH54-del (lane 1), Clone 7 at 100% of survival (lane 2) and aged G418 sensitive Clone 7 and its respective Southern Blot. From left to right the probes hybridize *ALG6* and *BRX1* near the centromere and *OPI1* on chromosome VIII that also hybridizes the spontaneous translocated chromosome. Red arrows shows the presence of the spontaneous translocated chromosome also in the G418 sensitive colonies. Red Circles highlight the presence of the translocated chromosome for non-chronologically aged Clone 7. The TrChromosome cannot be seen in the gel because its height is similar to chromosome XVI.

4.6. Loss of the Translocated Chromosome occurs when the cell re-enters the cell cycle.

A very simple experiment was designed in order to verify if the loss of the chromosome was happening after the cells were plated or when they were still in the depleted media. The same colonies that were found G418-sensitive were completely retrieved by carving them out of the YPD agar and their genomic DNA was extracted. After the concentration and the purity of the genomic DNA was quantified and diluted in order to have 30ng/μl for all the samples, 10 G418 sensitive colonies were analyzed by qPCR using as control 10 G418 resistant colonies. The result of the experiment showed that the G418-sensitive colony is populated to about 50% by cells having the Kan^R and therefore the TrChromosome (Figure 26). This clearly indicates that the loss is happening after the cells are plated on rich media and half way during the colony growth.

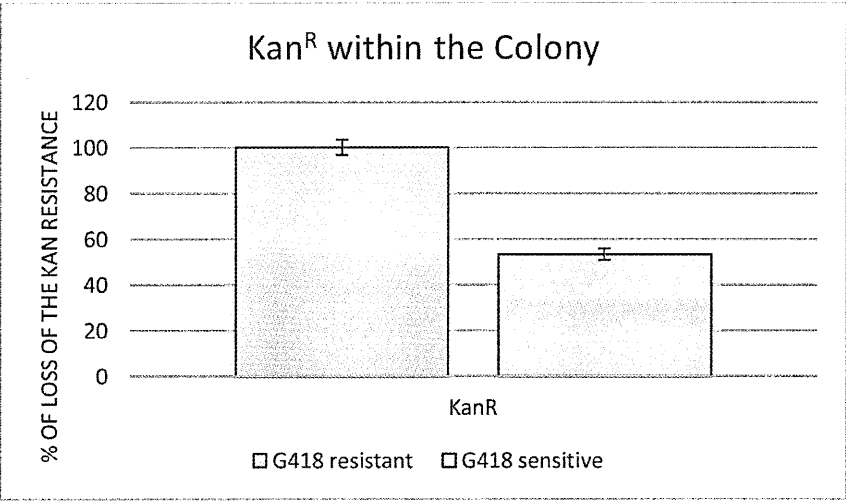


Figure 26: Graphic representation of the loss of the Kan^R within a single colony. Blue column represents the G418 resistant colony, which value was normalized to 100% and Red column represents the G418 sensitive colonies that were compared to the G418 resistant. The p value of the G418 sensitive, calculated with the Student T-Test is below 0.01. The percentage of loss was calculated by using the formula of the absolute quantification as described in the M&M and normalizing it accordingly. Ten single colonies were analysed independently for both the G418 resistant and the G418 sensitive.

4.7. The Gene interactions reveal a high level of genetic deregulation

After all the results described in the previous paragraphs it was interesting to analyze which genes could be responsible for the high ROS levels and the very different Life Spans of the Translocants. Using the already complete MicroArray Gene Expression analysis done on the strain D10-Big (Nikitin et al. in production), known genes functioning in the Oxidative Stress response and in the Mitochondrial Homeostasis were selected having at least a 2 fold gene deregulation. This list of genes: *PRX1, HOR2, BLM10, HSP12, CTT1, TRX2, YHB1, HSP150, SRX1, UTH1, OGG1, CCS1, GAD1, ALD6, GRE1* for the Oxidative Stress Response and *MDM10, SCO2, ATP3, AAC3, STF1, INH1, GGC1, EHD3, CYC7, PIC2, COX15, NCA3, QCR8, ATP2, MDM35, CYT2, HAP4, OAC1, COX17, ATP14, COX8, CAT2, CYB2, COQ5, AAC1, ISF1, ATP23, MIM1, MDM38, CRC1, MCT1, ODC1, YAH1, PDH1, SUE1* for the Mitochondrial Homeostasis were analyzed using the GeneMANIA algorithm server [87] in order to see their Physical and Genetic interactions.

For the Oxidative Stress genes network the algorithm showed that many other genes were indirectly influenced by the genetic deregulations with some known ROS resistance genes such as *SOD1* and *PRX1*. These results give interesting insights on the very high genetic deregulation that occur in translocant yeast cells and, in this case, how the huge unbalance of the Reactive Oxygen Species Homeostasis influences the levels of ROS at the end of the translocant chronological life span.

(Figure 27)

It is also interesting to note that all of the genes used in the query are not located on the chromosomes involved in the induced translocation (chromosomes XV and VIII).

The mitochondrial homeostasis genes were taken into account because, considering that D10-Big has a very short lifespan and is not able to grow on non-fermentable carbon sources, it is more probable that its short life span is due a respiratory malfunction and thereby the mitochondrial homeostasis genes were the obvious choice to take into analysis.

If we look at the Mitochondrial Homeostasis network we will see a much more complex interaction involving genes implied in yeast ageing such as *SIT4*, *PEX6*, *PHB1*, *YME1*, *UTH1*, and *PHB1*. This information correlates with the difference in life span exhibited by the translocants and how catastrophic a chromosome translocation event may be for a cell. (Figure 28)

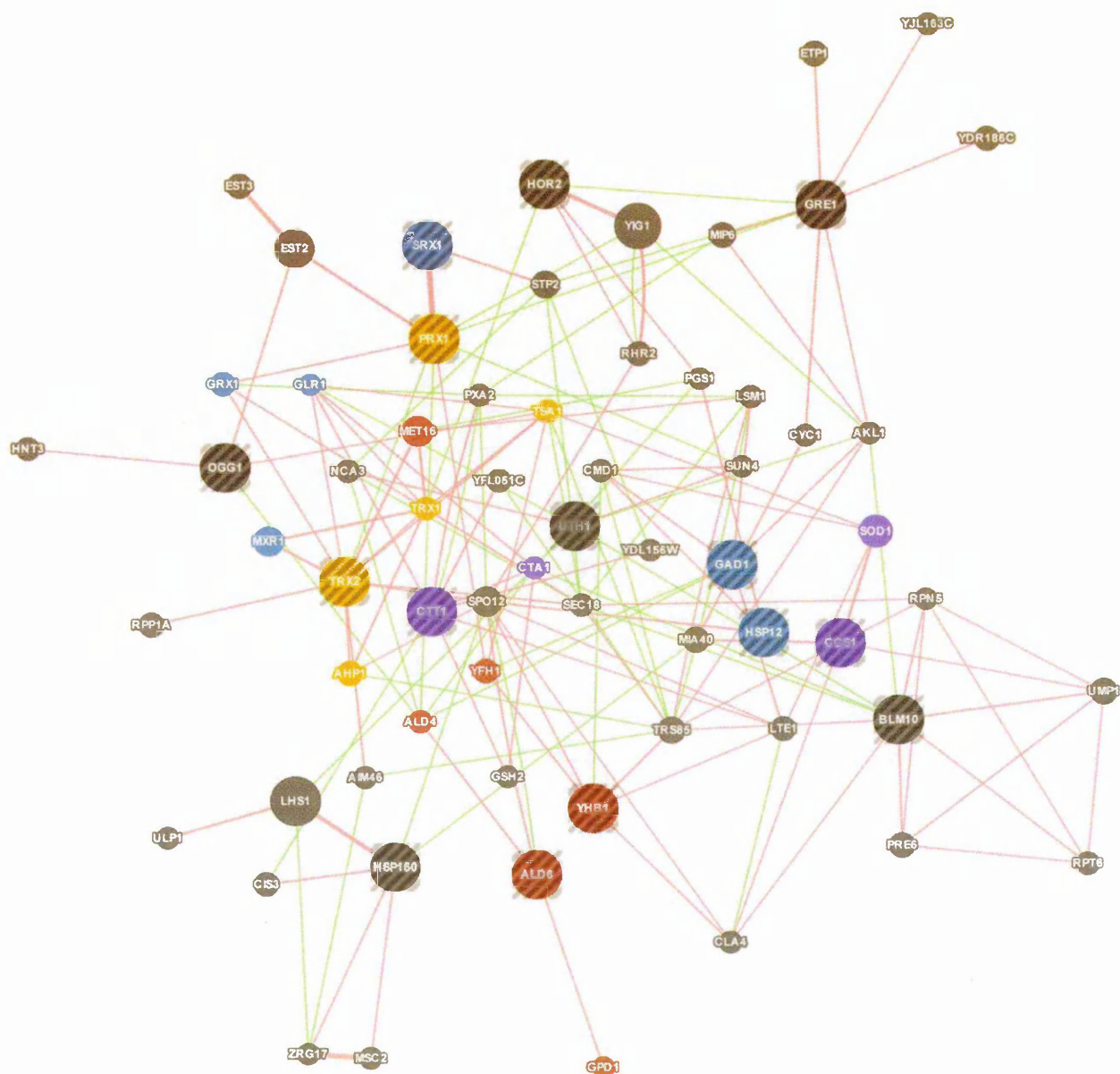


Figure 27: Oxidative Stress Response network. The genes with grey stripes are the ones used in the query. Purple defines the gene responsible for the Response to the Reactive Oxygen Species, Yellow for the Cell Redox Homeostasis, Blue is the Response to Oxidative Stress, Red for the Oxidoreductase Activity and Black are genes involved by other means with Oxidative Stress. Red Lines indicate Physical Interactions whereas Green line show genetic interactions. The size of the circles is directly correlated to the number of interactions.

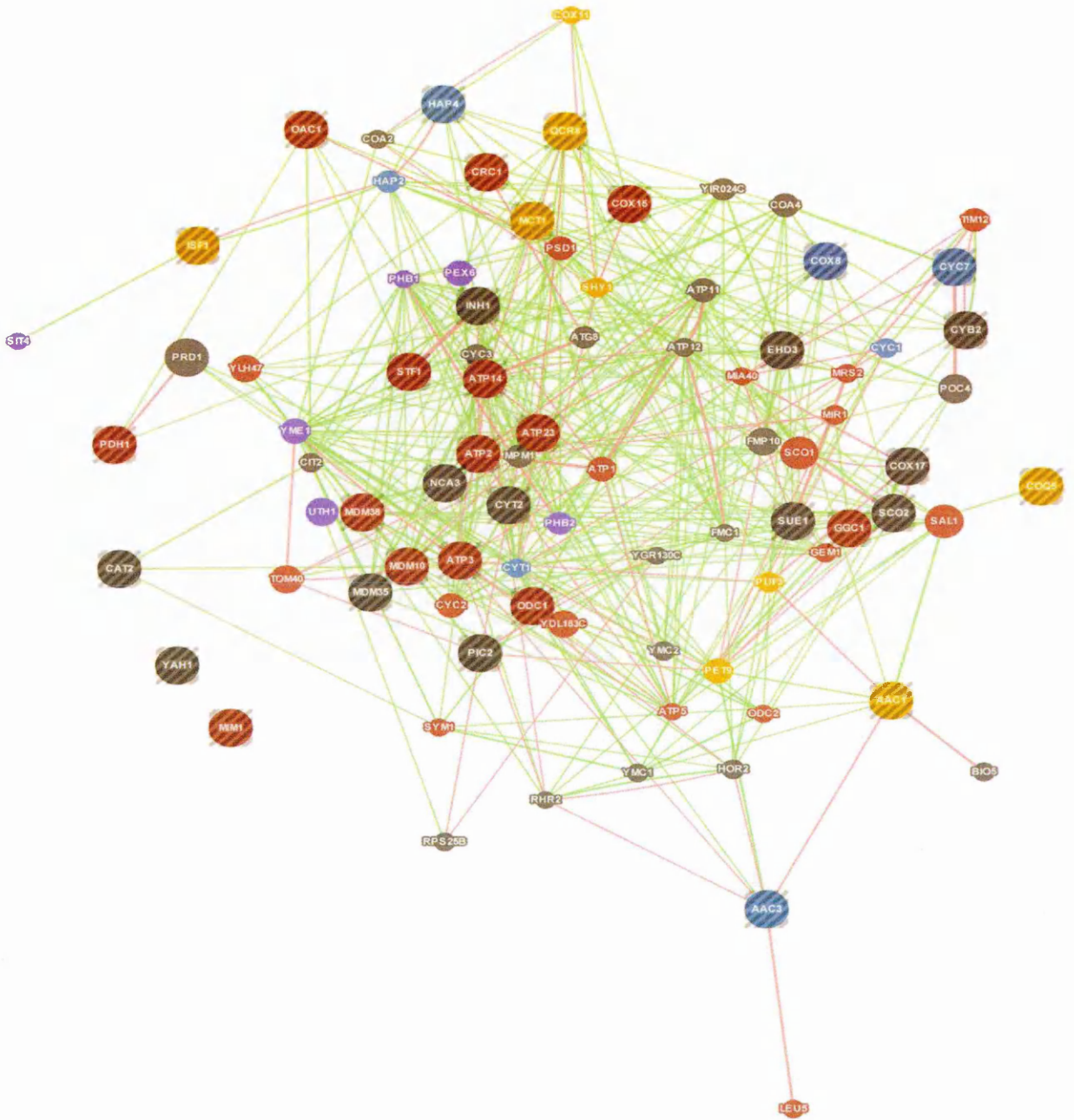


Figure 28: Mitochondrial homeostasis network. The genes with grey stripes are the ones used in the query. Purple defines the gene responsible for Ageing, Yellow for the Aerobic Respiration, Blue is for Cellular Respiration, Red for the Mitochondrial Membrane and Black are genes involved in the Mitochondrial Homeostasis. Red Lines indicate Physical Interactions whereas Green line show genetic interactions. The size of the circles is directly correlated to the number of interactions.

5. Discussion

The Bridge Induced Translocation technique developed in Bruschi's lab is an excellent method to induce a chromosome translocation by targeting DNA homologies within any part of the genome without pre-engineering the cell. This technique is much more than a simple method to induce a translocation but it may also be seen as a speciation inducer due to the high genetic rearrangement that the cells has to generate in order to adapt to the new condition. This hypothesis is very well demonstrated by the fact that the same bridge induce translocation gives different aneuploidies [54], different chronological life spans and different loss rates of the translocated chromosomes.

The different chronological life spans could be partially explained by, firstly the fact that more than 1000 genes are deregulated after the BIT event (Nikitin et al. in production) and this evolves in the different phenotypes shown by the translocants. Furthermore the short-lived strains D10-Big, D10-Small, Susu5 with the exception of Susu7 are unable to grow on non-fermentable carbon sources and have a very high gene deregulation of the mitochondrial homeostasis network, which progresses in the deregulation of also known ageing genes such as *SIT4*, *PEX6*, *PHB1*, *YME1*, *UTH1*, and *PHB1*. This could be a plausible explanation for their short life span since the inability to respire without the appropriate carbon sources makes them die very quickly during their CLS [88].

Interestingly, these life span variations between the translocants do not correlate with their reactive oxygen species levels which are relatively normal at the beginning of the life span, exception made for Susu7, but increase dramatically at the end of the life span reaching peaks of 14-fold increase. The interesting fact is that this increase in ROS levels was also found in the *rdh54Δ/rdh54Δ* strain that has formed a spontaneous translocation [53] after the *RDH54* double knock-out. This could be a demonstration to the fact that the increase in ROS levels at the end of the life span is directly linked to a translocation event but does not correlate with the life span since most of the translocants have a wild type like CLS. These data demonstrate that there is no direct connection between ROS levels and CLS and that most probably the translocants not only accumulate the reactive oxygen species but are also resistant to their oxidative stress, furthermore the increase in ROS levels could be linked to an increase in genomic instability that in contrast does not affect the yeast life span. This high level of adaptation is the solid point of the translocants demonstrating that chromosome translocation is much more than a simple movement of genetic material from one chromosome to another but actually an adaptation inducing event with all the consequences of the case.

Additional experiments on the ROS levels were done in order to see if these translocants were dying in an apoptotic manner in accordance with their CLS. Since it is known that, also in yeast, oxidative stress is a mediator of apoptosis and that by deleting *SOD1* and *SOD2*, the known yeast Superoxide Dismutases, you can enhance the chronological life span decreasing the levels of ROS [63, 86], the Annexin V with

Propidium Iodide experiment was performed at day1 and day 6 of the translocants CLS.

The results demonstrated that the translocants at the beginning of their life span have higher apoptotic rate than the wild type San1, and this is in accordance with the FUN-1 vital staining reported in the paper of Dr. Rossi [54], but with the increase of the number of days the long-lived Susu9 has a mild increase in cells entering either apoptosis or necrosis, whereas the short lived Susu5 and Susu7 have a dramatic increase in cell death comparable with their life span, with Susu5 preferentially entering necrosis. This is a further indication that the ROS levels, in this case, do not enhance the apoptotic rate of the translocants and demonstrate that these cells have adapted to a highly stressful situation.

The main focus of this work, though, is the stability of the translocated chromosome and since it has been demonstrated that these cells are highly adaptable it was of interest to understand if they would keep the induced translocated chromosome. An initial screening by replica plating the CFU, used for calculating the CLS, on G418 was done in order to select the cells that were G418 sensitive and that presumably had some rearrangement in their translocated chromosome. To actually prove the loss of the chromosome, after performing a Pulse Field Gel Electrophoresis in order to separate all the yeast chromosomes, specifically designed probes hybridizing near the TrChromosome centromere were used and showed that the chromosome as a structure was no longer present. Some hybridization, like in the case of D11, showed rearrangements of the TrChromosome and that there is the possibility that the TrChromosome could be entirely rearranged.

As demonstrated by the data, the loss is of different rates between the different sets of translocations (XVI-IX vs. XV-VIII) showing that most probably the translocation between *ADH1* and *DUR3* is much more stable than the translocation between *SSU1* and *SUC2* and that the size of the TrChromosome could have an influence on their stability. But the surprising result is that most of the cells kept their spontaneous translocation or chromosomal rearrangement, exception made for Susu7 that showed a high genomic instability. These results give us clues on the fact that maybe the translocants preferentially lose the induced translocated chromosome in contrast to a spontaneous translocation. To further evaluate this hypothesis the same experiments were performed on the wild type strain San1 with the *rdh54Δ/rdh54Δ* that has a spontaneous translocation between chromosome IV and VIII. In addition the very stable translocation between *ADH1* and *DUR3* was induced forming clone 4, clone 7 and clone 16. The results were consistent showing that the spontaneous translocation was kept, in all the G418 sensitive colonies, but the induced one was lost already at day 1 [53] exponentially increasing during the CLS.

It is important to note that the spontaneous translocation is always kept also in the absence of *RDH54* in contrast to the translocation XV-VIII that in the presence of *RDH54* is very stable but in its absence increases dramatically its loss.

The interesting fact is that the spontaneous TrChromosome loss that is seen in the translocants is not negatively affected by the size of the chromosome, most probably due to the fact that it may contain essential genes that need to be stoichiometrically coherent. In fact the rearrangements of Susu5 and of *RDH54-del*

that are smaller than the induced TrChromosome are kept in all the G418 sensitive colonies whereas the TrChromosome is lost. This is in contrast to the loss of the native chromosomes that seems to be negatively affected by the size with the only exception of chromosome III which is known to be highly recombinogenic due to its HMR, HML and MAT locus [92].

At this point the only additional answer needed was to understand where the loss was happening while the cells were still in liquid or after they were plated on rich media. The colonies that were G418 sensitive by replica plating, were completely retrieved from the petri dish and their genome extracted in order to perform a qPCR determining the gene copy number of the Kan^R. The results indicated that the colony is formed for its 50% by cells that still retain the Kan^R and by 50% without the resistance. The G418 sensitive colonies were compared to colonies grown exclusively on G418. This shows that the loss is happening when the cells re-enter the cell cycle.

This indicates that the cell, while it is chronologically ageing, accumulates age-dependent damage that will bring to the loss of the translocated chromosome only if the cells re-enter the cell cycle. Most probably the chromosome is lost during M phase due to a mis-pairing of the translocated chromosome.

Much can be discussed on the difference in the TrChromosome stability between the different translocants that derive from the same BIT event. Most probably it is due to the very different complexity of the aneuploidy established of the different strains [54] and consequently the unbalanced gene dosage of important stoichiometric proteins such as those of the spindle body assembly and those that

interact physically and functionally with the chromosome. In fact it has already been demonstrated that an unbalanced stoichiometry in specific proteins of the spindle body is sufficient to drive chromosome mis-segregation in cancer cell lines.

[93]

But there is to say that there is a direct correlation between Chromosomal Instability and aneuploidy, even if most yeast strains can produce stable disomic and trisomic derivatives and this refers also to human cells that can retain a stable aneuploidy status (e.g. trisomy 21 in Down Syndrome) [94]. It has been demonstrated that there is a high levels of chromosomal instability in yeast polyploids.[95] Note, however, that all human trisomies with the only exceptions of the sex chromosomes and chromosomes 21 and 13, are embryo-lethal.

This work shows that a genome that has stabilized after a chromosome translocation if put under the pressure of ageing has to re-adapt quickly and preferentially loses the non-essential chromosome which in the case of the translocants is the induced translocated chromosome in contrast to all the other genomic rearrangements that have formed due an adaptation of the cell to the BIT event. On this regards it is important to consider that the highest level of loss was seen in cells with a wide range of aneuploidies. In contrast, though, by simply deleting *RDH54* it was possible to increase the loss of the translocate chromosome XV-VIII, which does not form any aneuploidy. Furthermore the spontaneous translocation IV-VIII even in the absence of *RDH54* was maintained throughout the life span.

These results give a very first insight on how cancer-like cells behave when they age in a post-mitotic manner demonstrating that the event necessary for a cell to leave an apparently stable situation, is to re-enter the cell cycle as demonstrated by the fact that the loss of the chromosome is happening when the cells are plated on the rich media after ageing chronologically.

In conclusion this work illustrates how adaptable a highly unstable genome can be and how from the same event, in this case a Bridge Induced Translocation, cells can exhibit a different life span, apoptosis rate and chromosome instability but all retain the common feature of an enhanced ROS levels towards the end of the life span. All of this is due to the high genetic deregulation that affects not only the genes located on the chromosomes used for translocation but the entire genome (Nikitin et al. in production). In addition it also shows that the gene *RDH54* may play a role in the maintenance of the translocated chromosome [91] and in its correct segregation after ageing.

This thesis confirms once again that a chromosome translocation is much more than a simple movement of genetic material but it actually alters completely the cell in every aspect, and that Bridge Induced Translocation is an excellent method to induce in any part of the genome a translocation and promote adaptation and speciation.

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Table of Context

1. Introduction3

1.1. Cell cycle and chromosome dynamics3

1.1.1. Segregation.....4

1.1.2. Spindle Checkpoint8

1.2. Homologous Recombination.....11

1.2.1. Break Induced Replication.....14

1.3. Chromosomal Translocations.....17

1.4. Bridge Induced Translocation.....18

1.5. Ageing21

1.5.1. Chronological Ageing21

1.5.2. Oxidative Stress in Ageing cells.....24

1.5.3. Genomic Instability in Chronological Ageing yeast.....25

1.5.4. Chronological ageing and its relationship with Cancer27

2. Aim of the Thesis.....28

3. Materials and Methods29

3.1. Strains and Media.....29

3.1.1. Wild type strains and Translocants29

3.1.2. Media preparation30

3.2. Chronological Life Span assay and Replica Plating30

3.3. Pulse Field Electrophoresis and Southern Blot31

3.4. Table of Primers used for DIG labelled probes32

3.5. ROS Measurements33

3.6. Genomic Extraction and Gene Copy Number qPCR34

3.7. Annexin V staining35

4. Results37

4.1. Different Chronological Life Spans arise from the same Bridge Induced Translocations.....37

4.2. Reactive Oxygen Species levels of the translocants are not related to their life span and dramatically increase with the age of the population.42

4.3. Translocant cells exhibit higher apoptosis level at the beginning of their Chronological Life Span regardless of their ROS levels45

4.4. Loss of the translocated chromosome is loci-dependent and is preferred to the spontaneous translocation.....48

4.5. *rdh54Δ/rdh54Δ* translocants have a steady increase of loss of the TrChromosome. 55

4.6. Loss of the Translocated Chromosome occurs when the cell re-enters the cell cycle. 58

4.7. The Gene interactions reveal a high level of genetic deregulation.....59

5. Discussion63

6. References70

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Good Luck and Smile

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